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ASPECTS OF CONTROL OF PROTEASE ACTIVITY IN SOIL

by

GARRY R. COY

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Aspects of Control of Protease Activity in Soil", submitted by Garry R. Coy in partial fulfilment of the requirements for the degree of Master of Science in Soil Science in Soil Biochemistry.

DEDICATION

This thesis is dedicated to my best friend, lover, helpmate and mother of our two beautiful children, Shelagh and Michael, my wife Joan. She was my laboratory assistant at 3 a.m., counsellor in times of frustration and an echo of my conscience when the empty pages lay before me and my mind was filled with the siren call of the fields.

Abstract

Casein and several non-protein sources of C and N were used to examine mechanisms regulating protease activity in an Orthic Black Chernozem and an Orthic Gray Luvisol. Casein produced a significant but short-lived increase in protease activity in both soils used. Non-protein sources of C and N produced only minor changes in measured protease activity, and may be a measure of the shift in constitutive protease synthesis due to change in growth rate.

Attempts to induce protease synthesis with individual amino acids and dipeptides were unsuccessful. Ammonium, when incubated with the soil or added to the enzyme assay at levels up to 1000 $mg \cdot kg^{-1}$ soil, had no significant effect on exprotease synthesis and activity.

In the soils used in this study derepression by cyclic adenosine monophosphate (c-AMP) suggests that catabolite repression may be functioning to regulate exoprotease synthesis, although these results were statistically non-significant due to large data variance. Dibutyryl-c-AMP did not affect exoprotease synthesis. It is likely that two or more control mechanisms may be functioning independently in the heterogeneous soil system. The methods of activity measurement do not permit discrimination between the independent regulatory mechanisms. Future studies should be directed towards an understanding of multiple controls in heterogenous populations.

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to my advisor Dr. W.B. McGill. His guidance, encouragement, and patience were unflagging as I negotiated the tight curves and long hills of my studies. His objective approach and ability to think clearly when confronted with "muck and mystery" served, and will continue to serve, as an inspiration to me in this and future endeavors.

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ASPECTS OF CONTROL OF PROTEASE ACTIVITY IN SOIL

1. Introduction

Undecomposed plant residues and other organic materials added to soil are partly composed of large and complex polysaccharides and proteins. Decomposition of this raw material, and subsequent transformations that accompany the decay process, are primarily a function of microbial attack and secondarily a result of *in situ* chemical weathering and leaching. The large complex structures of raw organic matter must first be hydrolysed into progressively smaller units that can be absorbed by microorganisms. This is achieved largely by extracellular enzymes, produced by microorganisms and released into the extracellular environment. A variety of proteolytic exo-enzymes, of various substrate specificities and pH optima, have been identified in pure cultures. In soil, qualitative measurements of non-specific protease activity have been made and some pH optima have been established. These enzymes are grouped into neutral and alkaline proteases. Synthetic di-peptides, casein and gelatin have been used as substrates to measure soil protease activity.

Most studies to date, employing measurements of soil protease activity, have been either part of a broad spectrum enzyme activity characterization of the soil, or were designed to investigate relationships between enzyme activity and N mineralization rates. No reports are available of studies to determine if any of the mechanisms regulating protease synthesis by cells *in vitro* are expressed at the soil system level.

Amendment of previously air-dried soils with readily metabolized substrates has been shown to stimulate cell growth and division, accompanied by an increase in protease activity coinciding with the

period of rapid decline of viable cells. By implication the conclusion has been drawn, that the appearance of protease activity in soil is linked to cell lysis and release of cellular constituents into the soil environment.

In this study laboratory experiments using air dried and preincubated soils were carried out to determine if protease activity is affected by amendment of the soils with varying levels of readily metabilized C and N-containing compounds. Addition of casein to the soil as an amendment was used to examine aspects of control of synthesis of new protease enzyme, and compounds involved in intracellular control mechanisms in pure culture were added to soil to evaluate control of soil protease activity.

2. Literature Review

2.1 Laboratory studies

Proteolytic enzyme activities in soil were documented as early as 1910 (Fermi, cited by Skujins 1978). There have been numerous investigations of extracellular proteases in subsequent years and interest has increased dramatically in the last five years because the degradation of proteins is an important part of the nitrogen cycle in soils (Burns 1982). Much of the earlier work was primarily a survey of the measurable enzyme activities in soil and was carried out under conditions of *in vitro* assay. Extracted solutions from soil, or soil suspensions using buffered solutions were principally employed, and a variety of substrates were used (Burns 1978).

Recently emphasis has been placed on the kinetics and biochemical characteristics of enzymes, for which much less data is available concerning exocellular proteases in soil. Enzyme kinetic studies in soils are influenced by the heterogeneous nature of the soil system. In contrast to pure culture studies where environmental conditions and nutrient availabilities can be rigidly controlled (McLaren and Packer 1970). There are several sites of enzyme activity in soils ie; solution phase, adsorbed enzymes and those enzymes associated with cellular debris. Physical and chemical parameters of the soil environment affect the accessibility of the enzyme to the substrate, and ionic and covalent bonding of the enzymes to the soil and clay minerals influence the activity of the enzymes (McLaren and Packer 1970; Kiss *et al* 1975; Burns 1982). In addition, kinetic studies of enzyme activities in soil may be influenced by the existence, within the soil matrix, of several enzymes capable of catalysing the same reaction (Nannipierri *et al* 1982 b).

In general, enzyme kinetics in soil have been studied by means of exclusion techniques. Microbial proliferation during enzyme assays was excluded either by killing the biomass with gamma radiation, preventing microbial growth by using bacteriostatic agents such as toluene, or by employing short duration assays (Ladd and Butler 1972; Kiss et al 1975). Further refinements included selective substrates (Ladd and Butler 1972; Skujins 1978) and establishment of pH and temperature optima (Ladd and Butler 1972; Batistic et al 1980) for specific enzymes.

Exoprotease activity in soils can be separated into three broad groups on the basis of pH optima for maximum reaction velocity: the acidic, neutral and alkaline proteases. Most recent work has concentrated on the alkaline proteases. (Ladd and Butler 1972; Ross 1977; Nannipierri et al 1982 a,b).

Increases in protease activity frequently coincide with the period of rapid decline in viable numbers of bacteria (Ladd and Paul 1973). Nannipierri et al (1979) confirmed this observation. Implicit in this conclusion is the acceptance of a "cause and effect" relationship between the availability of a relatively large amount of protein and subsequent increase in protease activity. It would appear that the protein(s) are directly involved in triggering de novo protease synthesis.

Regulation of the synthesis of exoproteases has been studied extensively in vitro using pure culture methodologies employing defined growth conditions. Factors affecting exo-enzyme synthesis under these conditions have been reviewed by Glenn (1976) and will not

be detailed here. Specific regulatory mechanisms that have been documented for microorganisms *in vitro* are; catabolite repression (Bromke and Hammel 1978; Kimura and Tsuchiya 1982; Chaloupka *et al* 1982) endproduct inhibition (Hofsten and Tjeder 1965; May and Elliott 1968; Monboisse and Gouet 1979) and induced enzyme synthesis (Drucker 1973; Lasure 1980). Kimura and Tsuchiya (1982) demonstrated the existence of at least two different control mechanisms within the same genus of fungi.

From data detailing the regulatory mechanisms controlling exoprotease synthesis in both bacteria and fungi in pure cultures (Glenn 1976), it is suggested that in soil, two or more forms of independent control might reasonably be expected. A particular genus of microorganism cannot be isolated whilst keeping the soil system intact and thus observations of the expression of protease synthesis are restricted to system level measurements only.

In addition to the heterogeneity of the microbial population within a single soil sample, enzyme activities may differ widely among different soils (Kuprevich and Shcherbakova 1971). Thus differential expression of exoprotease regulation may occur.

3. Preliminary Observations and Development of Methodology

3.1 Introduction

There exists little information on the effects of the soil chemical and physical environment on the activities or production of protease enzymes. Ladd and Butler (1972) described a rapid and precise assay of soil alkaline proteases using dipeptide derivatives as substrates. In this assay system, the rate of substrate hydrolysis is proportional to the weight of soil used and the release of amino-N per unit weight of soil is directly related to the time of incubation. It is a convenient method for measuring activity of soil alkaline proteases.

An understanding of the role of soil proteases in turnover of organic matter in soil requires the knowledge of effects of changes in soil chemical environment on soil protease production and/or activity.

Protease synthesis requires an energy source and a N source, therefore, the intital objective was to determine if there was a relationship between availability of C and N and the production or activity of new proteases. The hypothese were that soil proteases: (1) are inducible; (2) respond directly or indirectly to changes in the microbial environment; and (3) demonstrate biological conservation as do other extracellular enzymes in soil-free systems.

In this first of a three part series of studies, experiments were designed to examine the effects of NH_4^+, casein, glucose and methyl xanthate (a nitrification inhibitor) alone and in combination, on protease production and activity in soil samples incubated under laboratory conditions. Ammonium was used because of its structural

similarity to the amino group in amino acids and also in part due to its relatively small size. Malhi and Nyborg, (1984) observed that when nitrification inhibitors are used to reduce the rate of conversion of NH_4^+ to NO_3^-, the mineralization rate of organic matter is also reduced. The build up of NH_4^+ due to nitrification inhibition, may regulate mineralization of organic matter possibly by the mechanism of end product inhibition.

Since ammonium-containing amendments were used, a modification of the assay system of Ladd and Butler (1972) using photometric determination of amino-N, was necessary. The development of a non-destructive ammonia removal method is described.

3.2 Materials and Methods

Soil

Soil for this study was collected from the Ap horizon of a fallow field on the Univeristy of Alberta farm at Ellerslie, Alberta. The soil was a Malmo silty-clay loam developed on lacustrine parent material (Appendix A). The samples were air dried and stored at room temperature (18°C) in 23 litre plastic containers with loose fitting lids.

The soil was ground with a mortar and pestle and passed through a standard 60 mesh brass screen. Roots and other plant material were removed manually.

Analytical Methods

All results were calculated on the basis of oven dry weight of the soil. Total carbon was measured by dry combustion using a Leco Induction Furnace.

Mineral-N was determined by steam distillation of a 2N KCl extract of the soil (McKeague, 1978).

Protease Activity

The methods used to determine protease activity were based on those described by Ladd and Butler (1972) using casein and the dipeptide derivative Carbobenzoxyphenylalanyl-leucine (CBZ-PL) as substrates. The procedure was modified by: (i) using an incubation period of two hours at a temperature of 40°C with horizontal shaking; and (ii) reacting casein substrate assays with ninhydrin as per CBZ-PL assays.

All values reported, except where otherwise indicated, are net activities determined by subtracting an appropriate blank either without substrate or without soil or both. Activity is expressed as mg of amino-N kg^{-1} h^{-1} based on a leucine standard.

Removal of Ammonia Prior to Colorimetric Determination of Amino Acid End Products

As described in the assay procedure of Ladd and Butler (1972) after stopping the reaction all samples and controls were centrifuged to give a particulate-free supernatant for amino-N determination. The following modifications were necessary to remove interference by ammonia.

Following centrifugation and prior to colorimetric determination an appropriate supernatant volume, (0.1-1.0 mL, depending on soil protease activity) was transferred to each of the required number of 150 x 25 mm test tubes. Sodium hydroxide (5M) was added quantitatively to each sample dropwise until a pH of greater than 10 was reached. In earlier trials the amount of 5M NaOH required for each of the several supernatant volumes (normal range of samples) had been determined (Appendix B). All samples were heated to dryness at 110°C in an oil bath. A volume of 5M HCl, equal to the volume of NaOH used, was then added to each tube to neutralize the NaOH, and sample volume increased to one ml with deionized water. Reaction with ninhydrin and colorimetric determination was carried out as described by Ladd and Butler (1972).

Km (apparent) Determination (CBZ-PL)

Following 14 days of incubation, unamended soils were assayed for protease activity with substrate (CBZ-PL) concentrations varying between 0.5 and 5.0 mM. A reciprocal plot of reaction velocity versus substrate concentration was employed to calculate Km (app).

Ninhydrin Reactive Nitrogen

Ninhydrin reactive nitrogen was measured daily in soil amended with casein and NH_4^+ during the 14 day incubation period. Ten g of soil (@ 30% moisture w/w) was shaken for 1h with 20 ml of deionized water.

The resultant slurry was centrifuged for 30 min at 2000 x g. Two 1 mL aliquots of the supernatant were reacted with ninhydrin as used during regular assays. Results were recorded as mg of amino-N kg^{-1} h^{-1} using a leucine standard.

Chemicals

All chemicals used were reagent grade with two exceptions. Methyl xanthate was prepared by Norwest Soils Research Ltd., Edmonton. The CBZ-PL was enzyme assay grade obtained from Sigma Chemical Company. Casein was obtained as sodium caseinate from B.D.H.

Soil Preincubation

Soil samples (300 g) were incubated at 25% moisture by weight in plastic containers having lids perforated to permit gaseous exchange. After a minimum period of 10 days preincubation the soil control (or unamended) pots were increased to 30% H_2O with deionized water. Pots designated to receive amendments were brought to 30% H_2O by dissolving the appropriate amendment in a volume of water necessary to achieve 30% H_2O by weight and mixing the solution into the soil sample.

Depending on the substrate used, 0.5 g of soil (CBZ-PL as substrate) or 1.0 g (casein as substrate) was removed in triplicate at intervals of 0,1,3,5,9, and 14 days and assayed for protease activity. Samples for assay on day 0 were removed 1h after thoroughly mixing the soil and amendment to allow for equilibration.

On all sampling days thorough mixing of the soil was performed prior to sampling to ensure homogeneity of sampling.

Soil Amendments

Unless otherwise indicated, casein, NH_4^+, KNO_3 and glucose were added as amendments on the basis of their respective C or N content; C at 1500 mg C kg^{-1} dry weight of soil, and mineral-N at 100 mg N kg^{-1} soil. Casein was added on the basis of a measured C content of 51% by weight. Methyl xanthate was added at 75 mg kg^{-1} soil.

Experimental Treatments

The folowing experimental treatments were used in this first series of studies.

(1) Control soil (no amendments)

(2) Glucose

(3) Glucose and NH_4^+

(4) Casein

(5) Casein and NH_4^+

(6) Glucose and Xanthate

(7) Casein and Xanthate

All treatments were assayed in triplicate for protease activity using casein and CBZ-PL as substrates.

To examine the inhibiting effect, if any, of NH_4^+, a separate series of assays were also conducted in the presence of NH_4^+ concentrations ranging from 0-400 mg NH_4^+-N kg^{-1} soil.

3.3 Results

Removal of Interferring Ammonia

There was a small decrease in the absorbance values after treatment to remove ammonia (Table 3.1) but the method clearly removed excess ammonia added to the samples either in the soil or in the assay. The method used and described earlier had little effect on the determination of a standard leucine sample or on the standard curve (Figure 3.1).

Effect of Substrate Concentration (CBZ-PL)

Several treatments were examined to determine if the relationship between assay substrate concentration and relative reaction velocity was consistent. Some evidence in the literature suggests there is a noticeable difference in apparent Km between a stabilized soil enzyme and its recently synthesized counterpart. Paulson and Kurtz (1970) reported a five-fold difference in K_m between "microbial" and "adsorbed" forms of urease activity in soil.

The relation between substrate concentration and reaction velocity was measured for several treatments. Figures 3.2 and 3.3 are plots of the data at t=14 days for the casein and glucose treatments respectively. The glucose treatment was not significantly different from the control (p=0.05).

The plots indicate that the enzyme pool in the soil receiving glucose as an amendment had a K_m apparent similar to that measured in the soil receiving casein as an amendment.

Table 3.1 Effect of Exogenous Ammonia Removal on Soil Protease Activity Measured as amino-N

NH_4^+ ($mg \cdot kg^{-1}$) in sol'n	Absorbance $_{570}$ Treated
0a	0.375 ± 0.021
33b	0.355 ± 0.022
33c	0.350 ± 0.019
66b	0.364 ± 0.024
66c	0.370 ± 0.022
166b	0.362 + 0.027
166c	0.355 ± 0.031

a - normal soil protease assay
b - ammonia added before assay
c - ammonia added after assay
An absorbance value of 0.038 was obtained in unspiked soil samples.

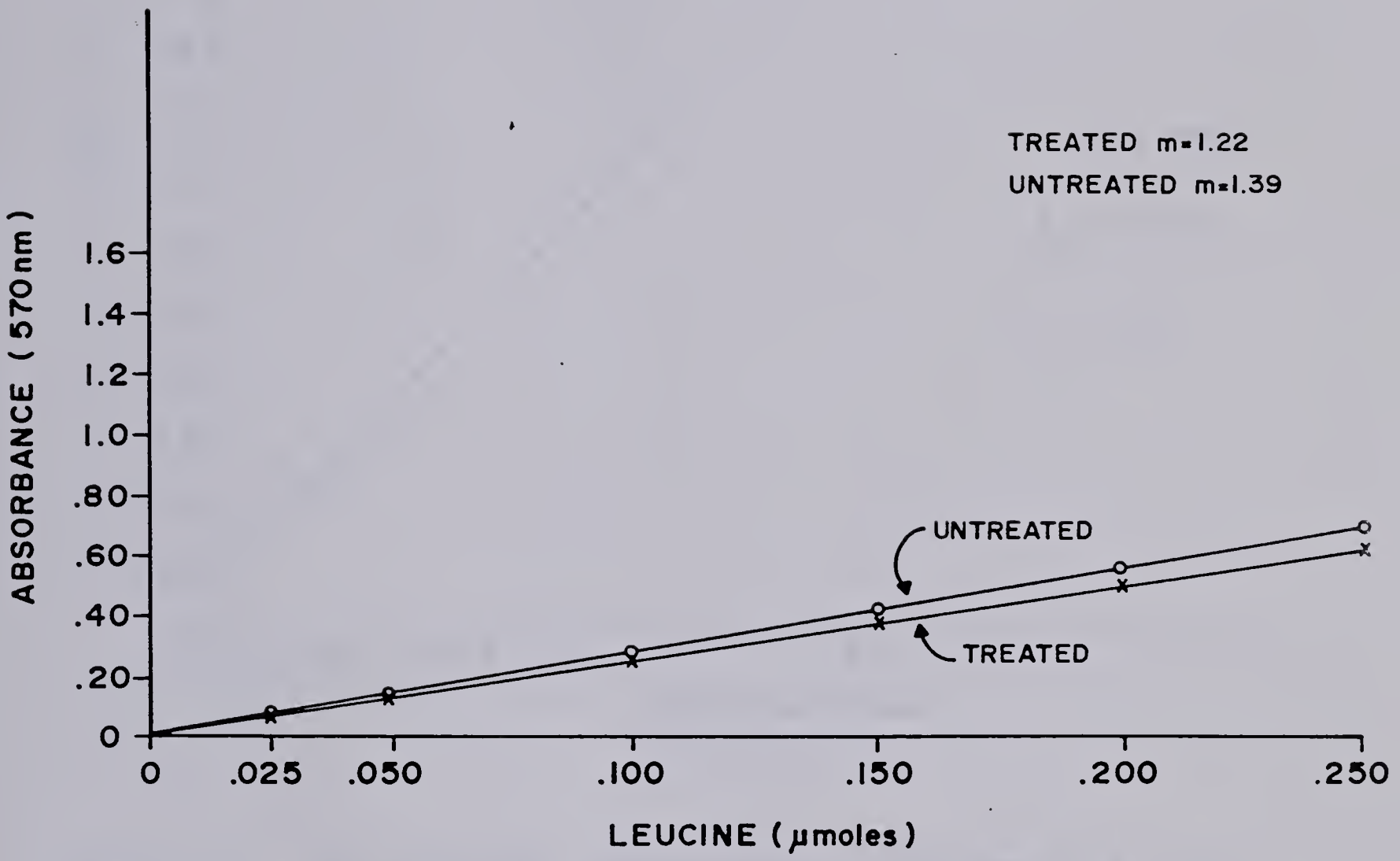

Figure 3.1 Effect of ammonia removal on leucine standards used to quantify protease activity.
Note that standards are shown as absolute quantities.
m = slope.

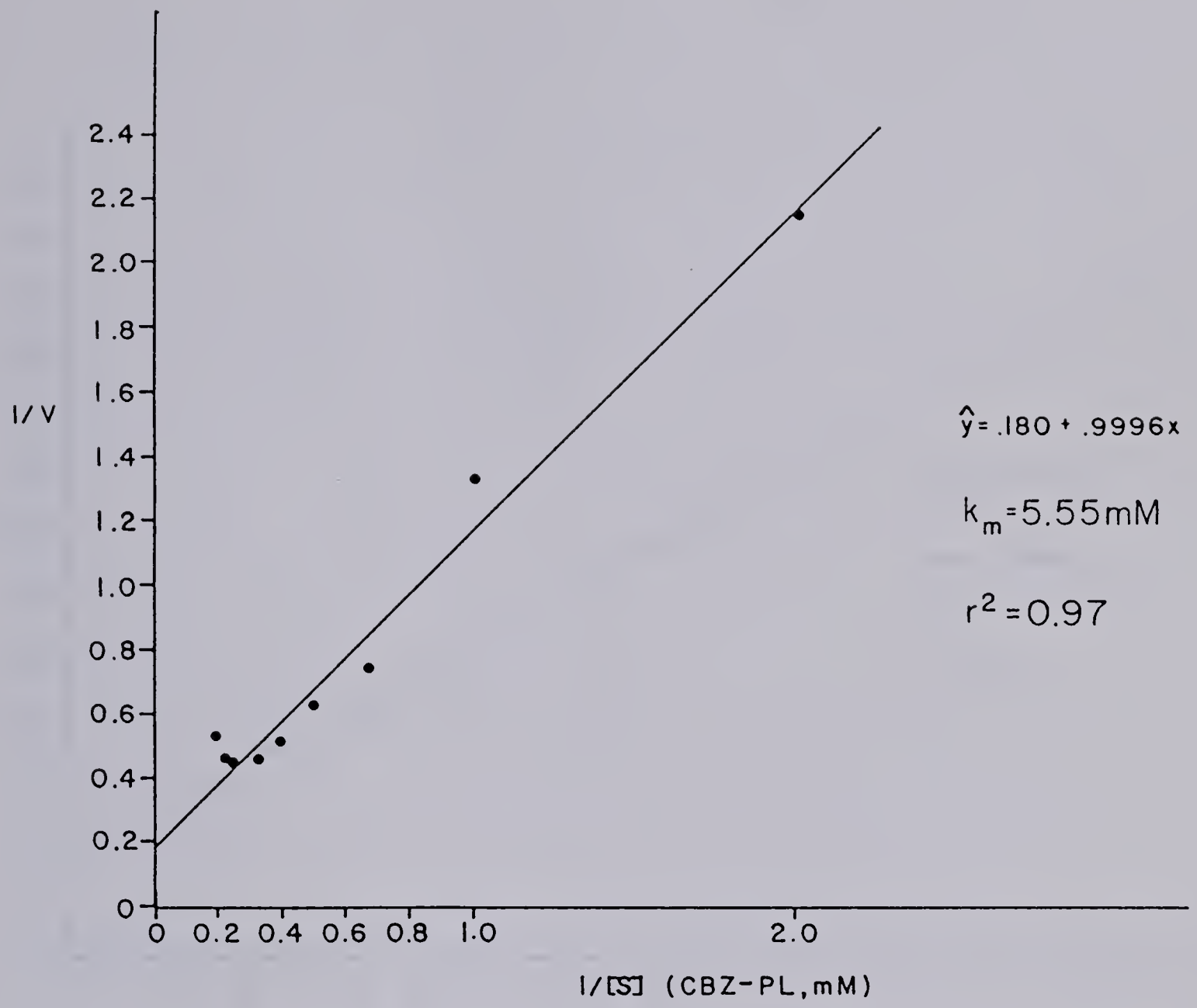

Figure 3.2 Km (apparent) determination of protease in a casein amended soil at t = 14 days.

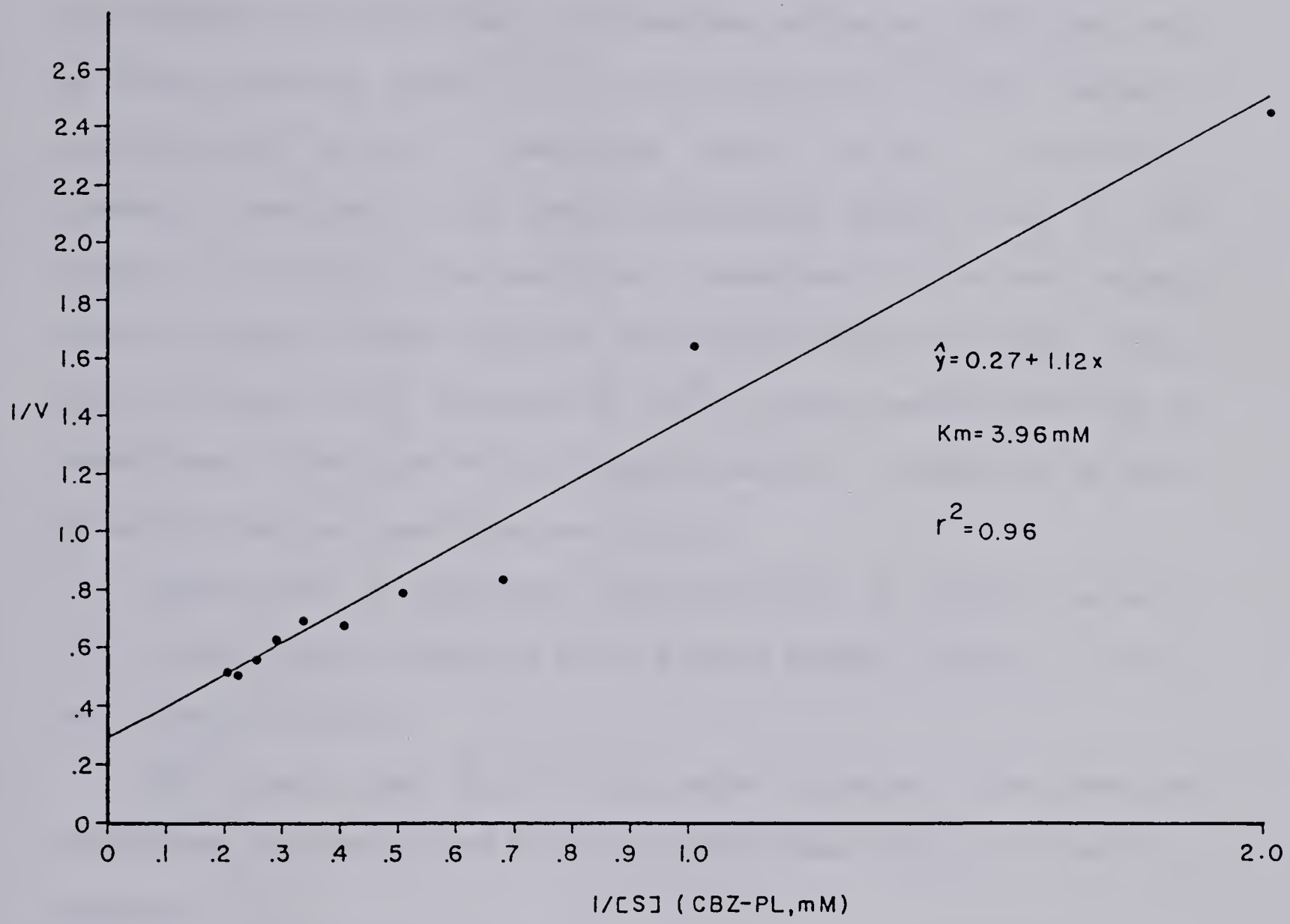

Figure 3.3 Km (apparent) determination of protease in a glucose amended soil at t = 14 days.

Effect of Amendment

Casein amendment increased measured protease activity by approximately five-fold when the dipeptide derivative CBZ-PL was used as assay substrate (Figure 3.4). The increase in activity reached a maximum level by day 3, declining rapidly by day 5, thereafter gradually levelling off to levels approaching those on day 0. The changes in activity using casein as a substrate in the assay showed similar trends to those observed when using CBZ-PL, but were always smaller (Figure 3.5). Addition of NH_4^+ to casein amended soil had no significant effect ($p=0.05$) on measured activity, regardless of what assay substrate was used (Figures 3.4,3.5).

Xanthate had no significant ($p=0.01$) effect on measured activity in a casein amended (Figure 3.6) or glucose amended (Figure 3.7) soil with either substrate.

When casein and NH_4^+-N were added together there was no significant difference ($p=0.01$) from casein alone (Figure 3.4 and 3.5, appendix).

Glucose added alone, or in combination with NH_4^+, gave results that were not significantly different ($p=0.05$) from the control.

Effect of NH_4^+ on Proteolytic Activity

In an attempt to examine whether NH_4^+ may be inhibitory to measured protease activity, the control soil and the glucose and casein amended soils were assayed separately in the presence of NH_4^+ at

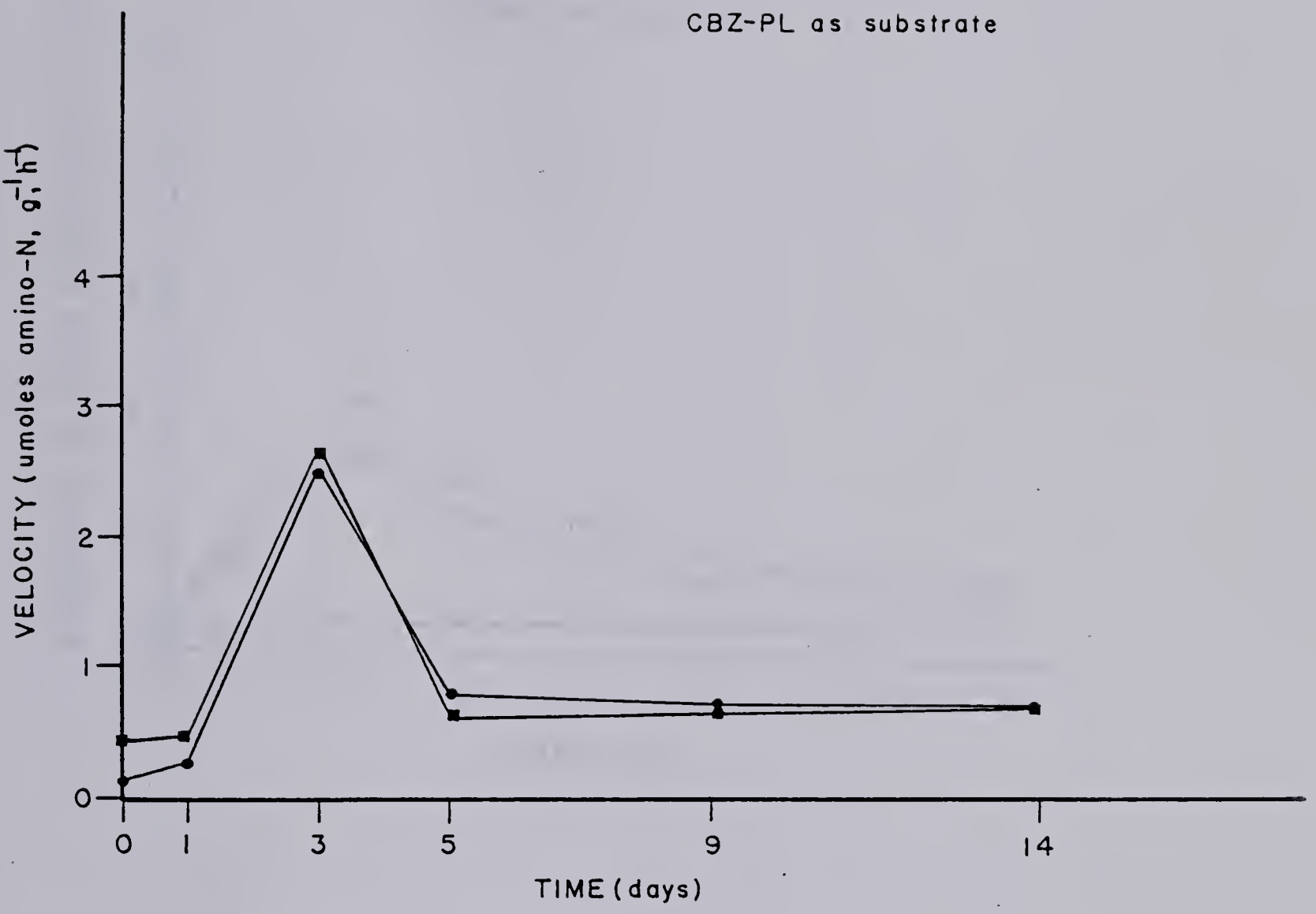

Figure 3.4 Effect of casein amendment on protease activity (measured with CBZ-PL as assay substrate) during incubation in the presence (■—■) and absence (●—●) of added NH_4 (400 mg $N \cdot kg^{-1}$ soil).

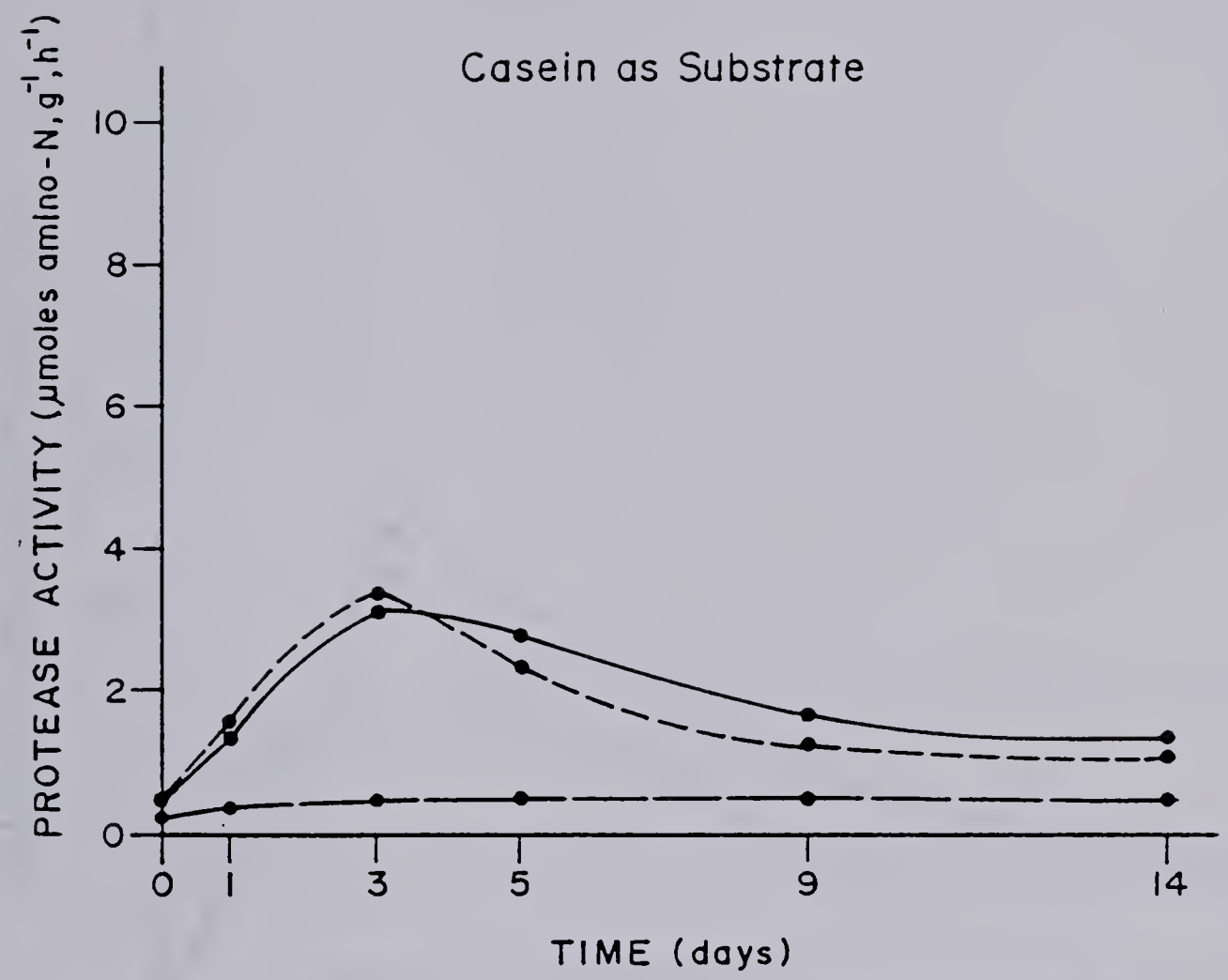

Figure 3.5 Effect of casein amendment on protease activity (measured with casein as assay substrate) during incubation in the presence (———) and absence (- - - - -) of added NH_4 (400 mg $N \cdot kg^{-1}$ soil).

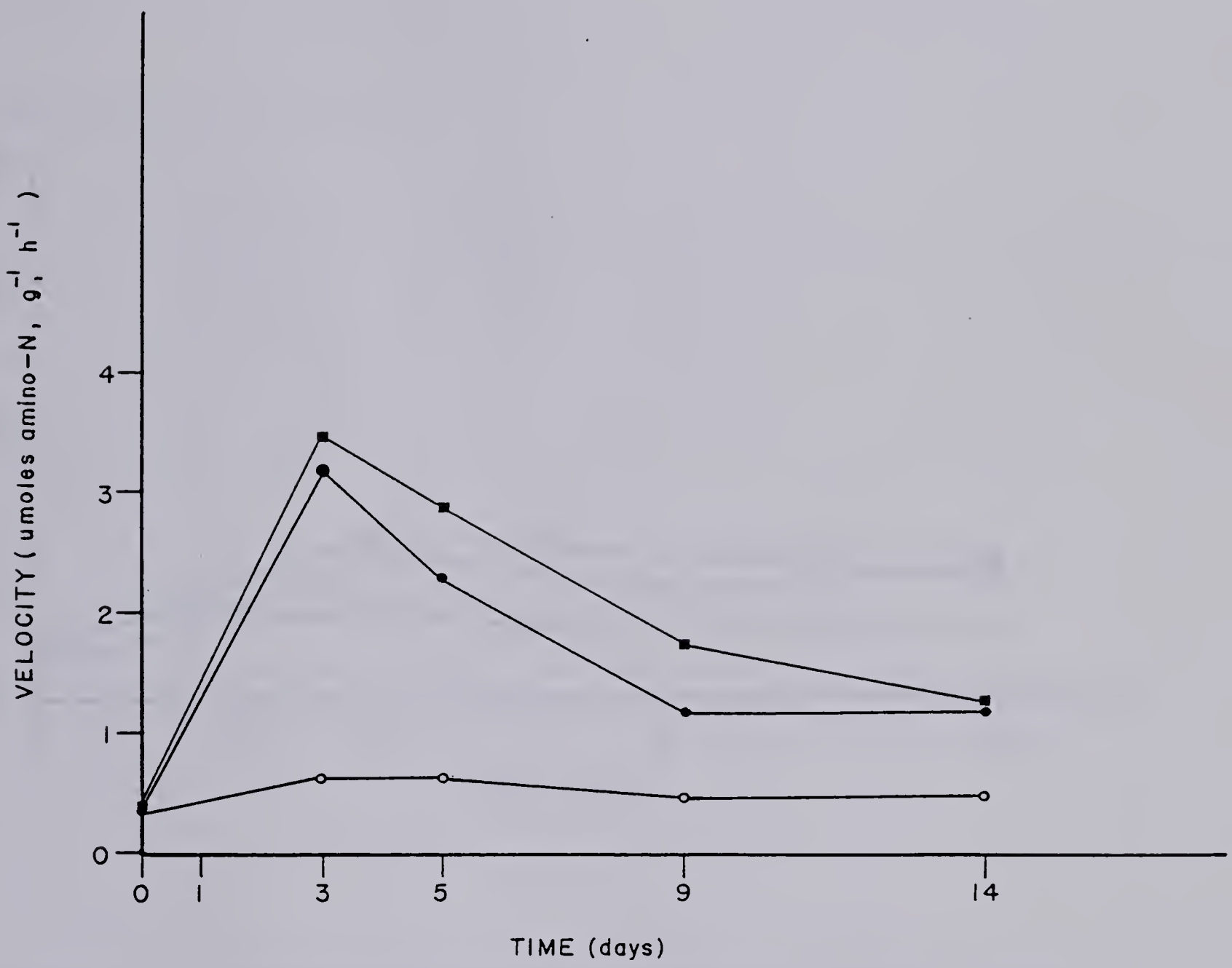

Figure 3.6 Protease activity of a casein and xanthate amended Malmo soil. (■—■) casein and xanthate; (●—●) casein (O—O) control.

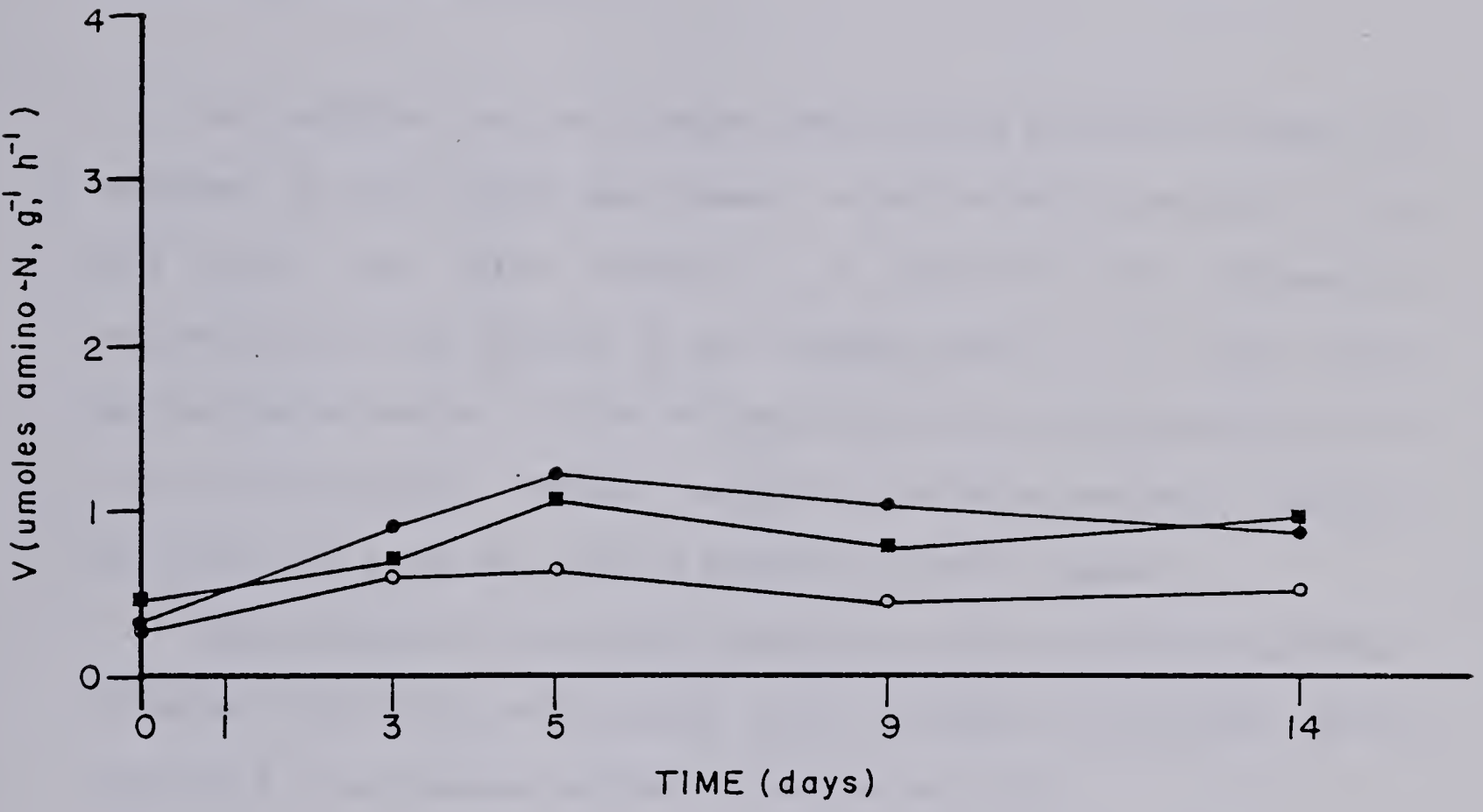

Figure 3.7 Protease activity of a glucose and xanthate amended Malmo soil. (■—■) Glucose; (●—●) Glucose and Xanthate; (○—○) control.

concentrations up to 400 mg kg^{-1} in solution (Figure 3.8). No clear trends in changes of activity were observed for any of the treatments tested.

Ninhydrin Reactive Nitrogen

The ninhydrin reactive nitrogen (NRN) values plotted in Figure 3.9 represent the unit weight measurement, of all soluble compounds in the soil that react with ninhydrin, as used in the photometric determination of end product in the protease assay. At no time during the incubation period did the NRN values duplicate the measured values of protease activity. Further, the pattern of distribution of NRN does not follow the trend of measured protease activity values.

The treatment to remove NH_3 described earlier, allows measurement of amino-N containing end products in the presence of relatively large quantities of extraneous ninhydrin reactive material.

3.4 Discussion

It has been previously shown that proteases in soil can hydrolyse proteins added to soil (Kiss et al 1975) as well as indigenous proteinaceous components of the soil ie; plant and animal debris. The results of this study have shown that protease activity in soil increases following the addition of proteins such as casein.

Soil samples receiving casein additions several days prior to being analyzed in the absence of an assay substrate gave results consistent with casein-free controls. Samples containing assay

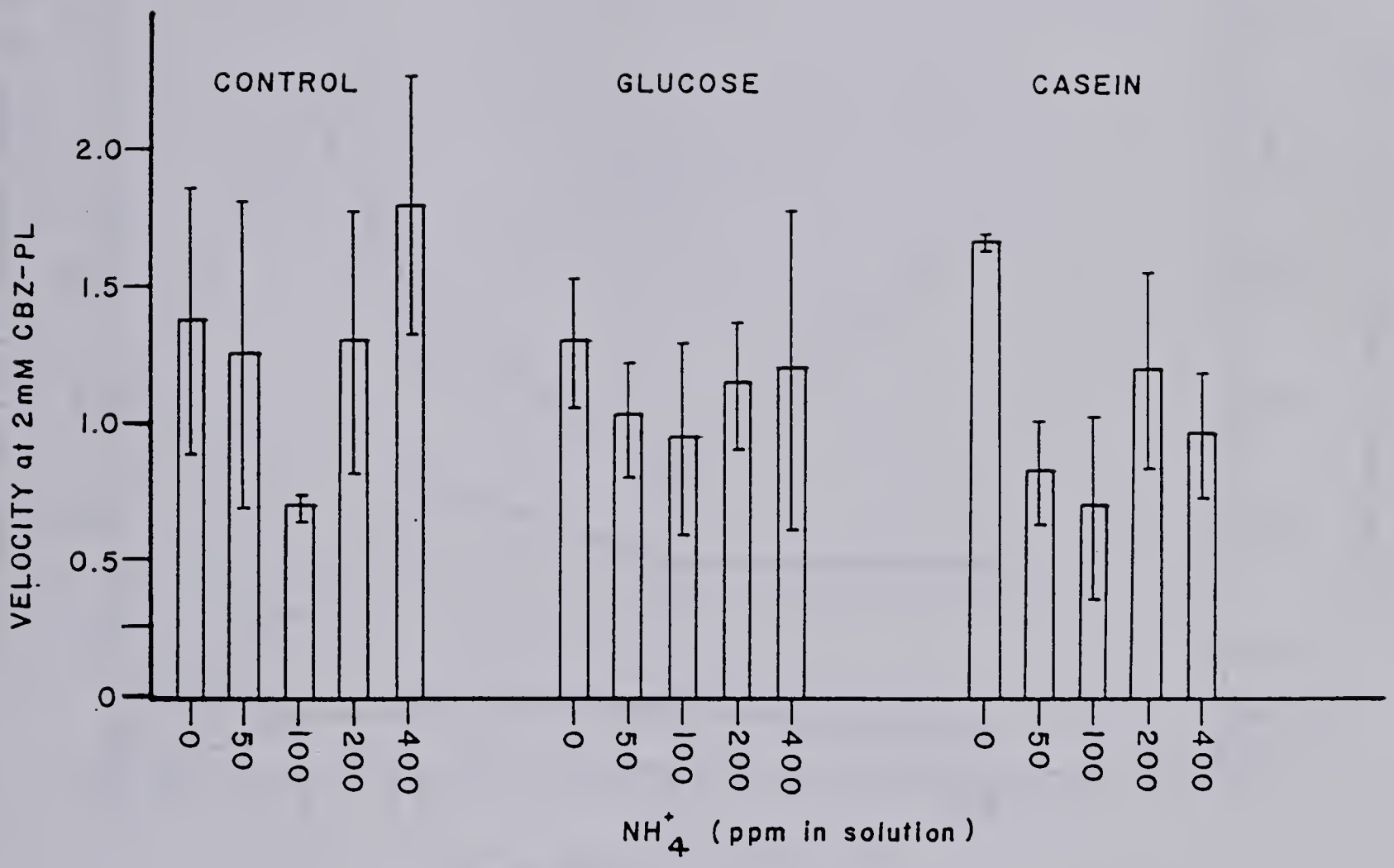

Figure 3.8 Effect of varying NH_4^+ concentration during assay on reaction velocity of protease in Glucose and Casein amended soils at t = 14 days.

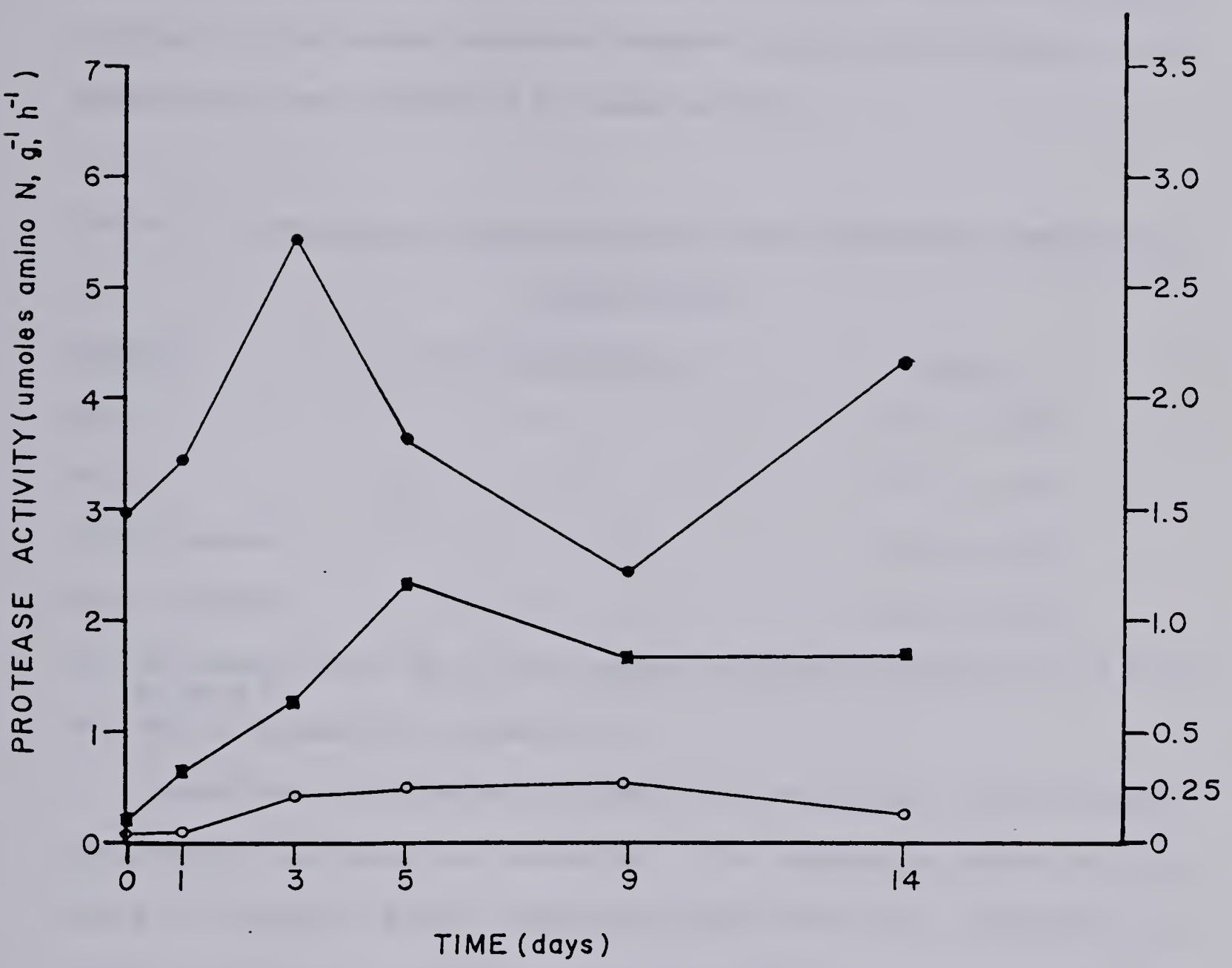

Figure 3.9 Comparison of protease activity and ninhydrin reactive nitrogen (NRN) values in a soil amended with casein and ammonium sulphate using CBZ-PL (●—●) and casein (○—○) as assay substrates. Initial NH_4^+ concentration = 100 ug, g^{-1}.

substrate showed consistent changes in activity, whereas substrate-free blanks did not (Table 3.2). Therefore the assay system was not an artifact of the casein amendment present in the soil but was in fact measuring the end product of protease activity.

Table 3.2 Comparison of Protease Assay Values from Casein Amended and Control Soils

Sample(a)	assay substrate(b)	Abs_{570nm}
Soil	+	0.25 ± 0.003
Soil	-	0.17 ± 0.017
Soil + casein	+	0.26 ± 0.026
Soil + casein	-	0.17 ± 0.010

a - all samples made up to 3 ml reaction volume by addition of 0.1 M tris Cl-pH 8.0.
b - CBZ-Pl, present (+), absent (-).

There was a consistent difference in the maximal values reached with CBZ-PL and casein as substrates. This observation indicates that there is either, a marked specificity for CBZ-PL as a substrate over casein, or, that substrate accessability is different for the two substrates used. The latter alternative is suggested by data of Kiss et al, (1975) who reported that the specific activity of proteases from soil extracts is increased over that of the unextracted soil, indicating a tortuosity effect, or diffusional constraint on the substrate reaching the enzyme, as described by Engasser and Horvath (1973). Diffusional and tortuosity constraints would be expected to affect casein more than CBZ-PL.

An increase in protease activity may be due to increase in specific activity of the enzyme or increase in the quantity of the

enzyme. It is important to be able to differentiate between the two phenomena and differentiate between the production of new enzymes as a result of proliferaton of protease producing microorganisms, and the production of new enzymes by a stable population, as a function of a stimulatory signal.

Research at the cellular and molecular level has repeatedly shown that a phenomenon, commonly referred to as biological conservation, exists in most biological systems to reduce energy demands and conserve materials required for synthesis and metabolism (Davis et al. 1973). It seems probable that an energy demanding function such as the production of extracellular proteases would be under some form of regulation with respect to the amount synthesized and the timing of synthesis, and that such regulation would be expressed in a discernible manner even in mixed populations such as exist in soil.

The addition of glucose and ammonia provides the microbial population with a C and a N source. From the abundant data in the literature it is certain that an increase in numbers of viable organisms results, but no significant increase in protease activity was observed when glucose, or glucose and ammonia were added to the soils used in this study (appendix). A significant increase in protease activity was observed only with the addition of casein.

Addition of ammonia, both in the incubated soil and during the assay, neither stimulated nor inhibited protease activity. These results are supported by the conclusions of Ross (1977), who also suggested that if the microbial population continuously synthesizes new protease enzyme, then high levels of NH_4^+-N might have a repressive effect on synthesis of new enzymes: However was unable to demonstrate

any repressive effect of NH_4^+-N on synthesis of protease. The results of this study and those of Ross (1977) and others indicate that ammonia is not directly involved in the regulation of protease synthesis or activity as expressed in soil systems.

Ladd and Paul (1973) reported an increase in alkaline protease activity by adding glucose and NO_3^--N to air-dried soils. The net difference between a control soil, wetted only with distilled water, and the treated soils amounted to a peak activity value of some 5 mg leucine equivalents kg^{-1} h^{-1}. The peak of activity observed by Ladd and Paul (1973) was similar in magnitude to the results of this study when using casein as an amendement but not when using glucose and ammonia. The peak (day 5) does not, however, appear at the same time as the peak of activity observed in this study, which appears on day 3 of the incubation period. This apparent conflict of results as a function of treatment response was examined and is reported in sections 4 and 5. Possibly the differences in results were due to soil physical and/or chemical characteristics.

It is essential that the changes in measured activity are not related to assay substrate concentration, and also that the reaction time and temperature of incubation will yield significantly measureable amounts of reaction product. Examination of reaction rate versus assay substrate concentration indicated that the assays conducted were within the linear reaction velocity range for the enzymes studied.

The four-fold difference in K_m (apparent) between the casein and glucose treatments is probably due to the existence of two distinct enzyme moieties; the adsorbed enzyme complex (covalently or ionically bound to humic components) and the newly synthesized enzyme group. Because no increase in activity was observed for the glucose amended soil it is thought that the higher K_m (app) observed reflects the lower affinity (or accessibility) of the adsorbed enzyme complex. The lower K_m (app) observed in the casein amended soil may reflect the activity of protease enzyme synthesized de novo.

Xanthate is a nitrification inhibitor that blocks the general reaction $NH_4^+ \rightarrow NO_2^- \rightarrow NO_3^-$. Xanthate had no measureable effect on protease synthesis or activity at the rates used in this study. This is additional evidence that NH_4^+-N and NO_3^--N are unlikely to be directly involved in the regulation of protease synthesis.

Earlier work in this study to remove interferring ammonia utilized a boiling water bath technique. This method had a major drawback because the volatilized ammonia dissolved in recondensed water at the top of the test tube. Use of an oilbath and heating the samples to dryness overcame this problem. The method used in this study was capable of removing all free NH_4^+-N from the samples. A very small decrease in absorbance (570 nm) values for both the standards and the samples was observed. This was likely due to a small amount of pyrolysis during drying. With fixed time of heating and with strict temperature control, the variance between heated and unheated samples become negligible, and more importantly, constant.

3.5 Summary

Protease activity in the soil used in this study increased following the addition of casein, but was not altered significantly by glucose or ammonia amendments used either in combination or separately. It is inferrered that regulatory mechanisms controlling production of extracellular protease activity in soil are linked to the presence of a protein or proteins, or parts thereof.

The results of this study corroborate, in part, the work of Ross (1977) and others, and show no stimulation or inhibition of protease activity with varying levels of NH_4^+-N. Thus the regulatory mechanism(s) are unlikely to be involved directly with changes in the NH_4^+-N levels in the external environment of the cell.

4. Factors Affecting Protease Activity

4.1 Introduction

Materials that provide an energy source or a source of microbial metabolic requirements or both, will, when added to soil stimulate microbial growth causing an increase in cell numbers (Shields 1972 Nannipierri et al, 1975) and subsequently constitutive enzymes and metabolites (Mandelstam and McQuillen, 1976). Inducible exoenzymes require an interaction of inducer molecule(s) with the cell-genetic material to initiate synthesis of a specific exoenzyme (Glenn, 1976 Mandelstam and McQuillen 1976; Glenn, 1976). Earlier work in this study suggests that protease activity measured in Malmo soil amended with casein is subject to a form of enzyme synthesis regulation that resembles in expression an inducible system because cell proliferation alone does not appear to produce a significant increase in measured protease activity.

In the first section of this study results were reported which showed a positive, but short-lived, enzyme activity response when casein was added to a preincubated soil. Further to this NH_4^+ added to the soil at concentrations up to 400 mg kg^{-1} did not appreciably affect this initial observation. These results prompted the following question: Was the activity response specific for the soil used, or would other soils display the same characteristics, and if so, to what extent? The hypothesis to be tested was soils low in organic matter normally have lower microbial population numbers associated with them so that activity responses would be similarly reduced. Two soils were used in subsequent studies to examine this question: one the eluviated Black Chernozem (Malmo, SiCL) used previously, and an orthic Gray Luvisol (Breton SiL) which had significantly lower organic matter

content and total nitrogen (appendix A).

Because Ladd and Paul (1973) did not use preincubated soils in their studies, a series of experiments was included to examine non-preincubated soils with respect to measured protease activity as a function of several C and N containing amendments. Amendments included all those previously used with the exception of casein alone, as well as glucose and NO_3^--N at two levels of carbon addition.

Initial studies indicated no stimulatory or inhibitory effect of ammonia at concentrations up to 400 mg kg^{-1}, therefore it was decided to use a higher concentration (1000 mg kg^{-1}) and repeat the experiments using two soils to overcome effects of NH_4^+ adsorption by soil colloids. NH_4^+-N amendments were added either to the soil as part of a treatment incubation, or to the assay.

4.2 Materials and Methods

Soils: The Malmo soil used in this series of experiments was described previously, (see also appendix A).

The Breton soil was collected from the Ap horizon of a summerfallowed field at the University of Alberta plots (NE-25-47-4 W5) at Breton, Alberta. The soil was an orthic Gray Luvisol developed on lacustro-till parent material (see appendix A).

Both soils were air dried at room temperature and stored in 23 litre metal containers with loosely fitting lids. Storage period did not exceed one year.

Soil Preincubation and Handling

The two soils were either preincubated @ 25% H_2O as previously

described (Materials and Methods, part I), or were brought to the desired moisture content immediately prior to the experimental sampling period by introducing the appropriate volume of water with a pipet, allowing the soil to equilibrate, and then mixing by hand.

Experimental Treatments

Series I (Preincubated soils) The following amendments were used at the indicated rates of C and/or N addition.

1) Control soil (no amendments)
2) Casein (1500 mg C kg^{-1}).
   NH_4^+-N (as $(NH_4)_2SO_4$ @ 100 mg N kg^{-1}.
   glucose @ (1500 mg C kg^{-1}).
3) casein and NH_4^+-N (as for 2 above)
4) casein and NH_4^+-N (C as for 2 above) N @ 1000 mg kg^{-1}.
5) glucose and NH_4^+-N, (C @ 1500 mg kg^{-1}, N @ 100 mg kg^{-1}).
6) glucose and casein, (both @ 1500 mg C kg^{-1}).

Series II (no preincubation)

1) Control soil (no amendments)
2) glucose and KNO_3, (C @ 1500 mg kg^{-1}, N @ 100 mg kg^{-1}).
3) glucose and KNO_3, (C @ 3000 mg kg^{-1}, N @ 100 mg kg^{-1}).
4) glucose (C @ 3000 mg kg^{-1}).

For series I soils amendments were added after a period of preincubation as previously described (Materials and Methods, part I). All treatments were assayed in triplicate using CBZ-PL as assay

substrate. Series I soils were also assayed using casein.

Soils receiving amendments under Series I treatment #2 were further examined by removing an additional triplicate set of samples and adding to them NH_4^+-N (as ammonium sulphate solution) to achieve a final concentration of 1000 mg N kg^{-1} immediately prior to assaying for protease activity.

4.3 Results

Series I Preincubated Soils

Results for the preincubated, nonamended (control) soils indicated that protease activity: (i) increased slightly due to the addition of water at time zero; (ii) was greater in the Malmo than the Breton soil; and, (iii) was greater with CBZ-PL compared to casein in the Malmo soil (Figures 4.1, 4.2).

Addition of casein along with glucose and NH_4^+(treatment #2) in both soils resulted in significant increases in measured protease activity that paralleled earlier data (part I, results) for casein alone or casein and NH_4^+ (Figures 4.3, 4.4). These data also confirm previous results that showed reproducible differences in the magnitude of the response, as well as a difference in the time of appearance of the peak values, between the two assay substrates CBZ-PL and casein.

When both soils were amended with casein and NH_4^+ the results for each soil were not significantly different for the two levels of NH_4^+-N used (Figures 4.5, 4.6) (appendix). Both soils with casein at both levels of NH_4^+ (Treatment 3 and 4) were significantly different from the control ($p=0.05$).

Amendment of both soils with glucose and NH_4^+ did not produce significantly different results from the control (Figures 4.7, 4.8).

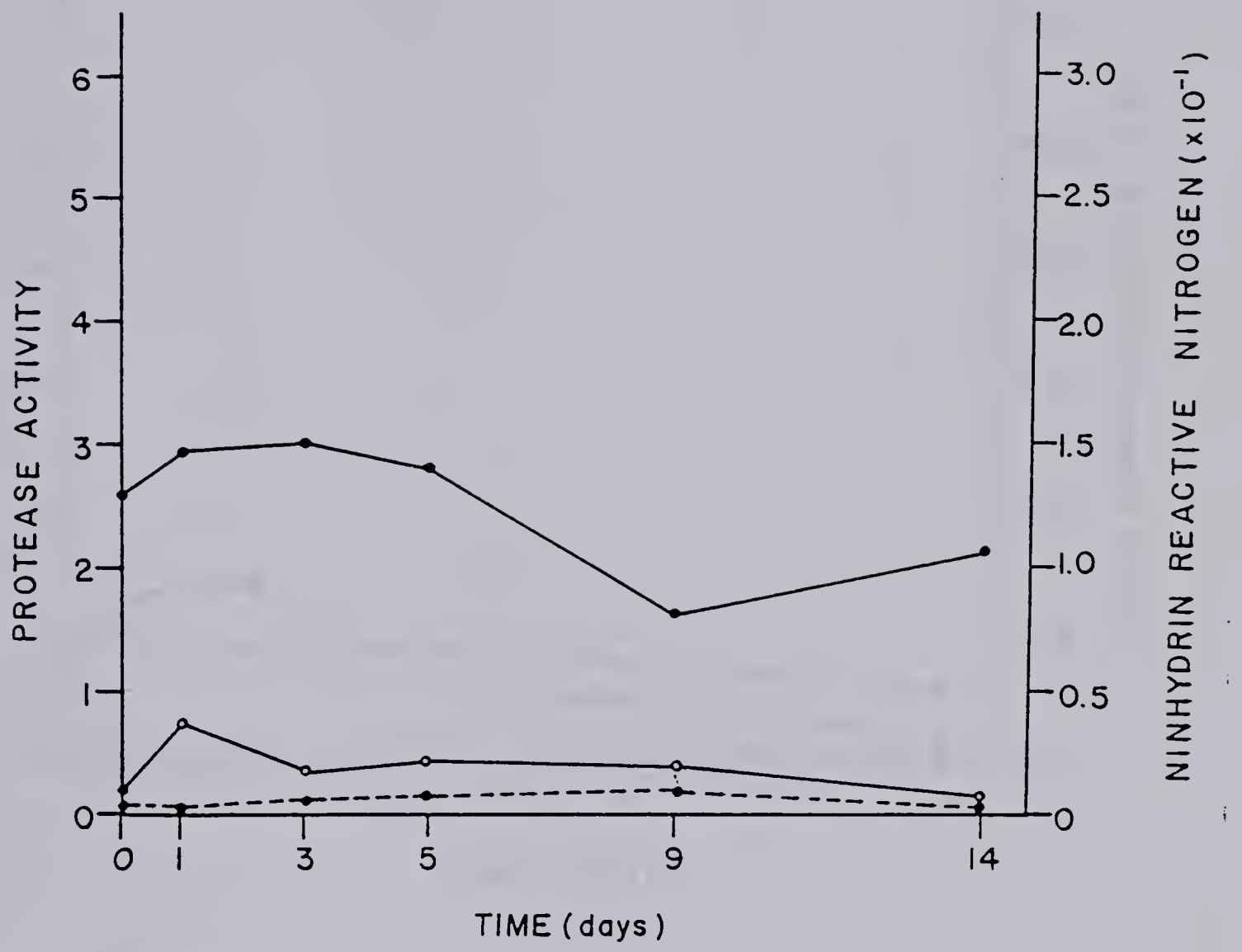

Figure 4.1 Protease activity in a preincubated nonamended Malmo soil. (●—●) assay substrate; CBZ-PL. (O—O) assay substrate; casein. (●--●) N-R-N.

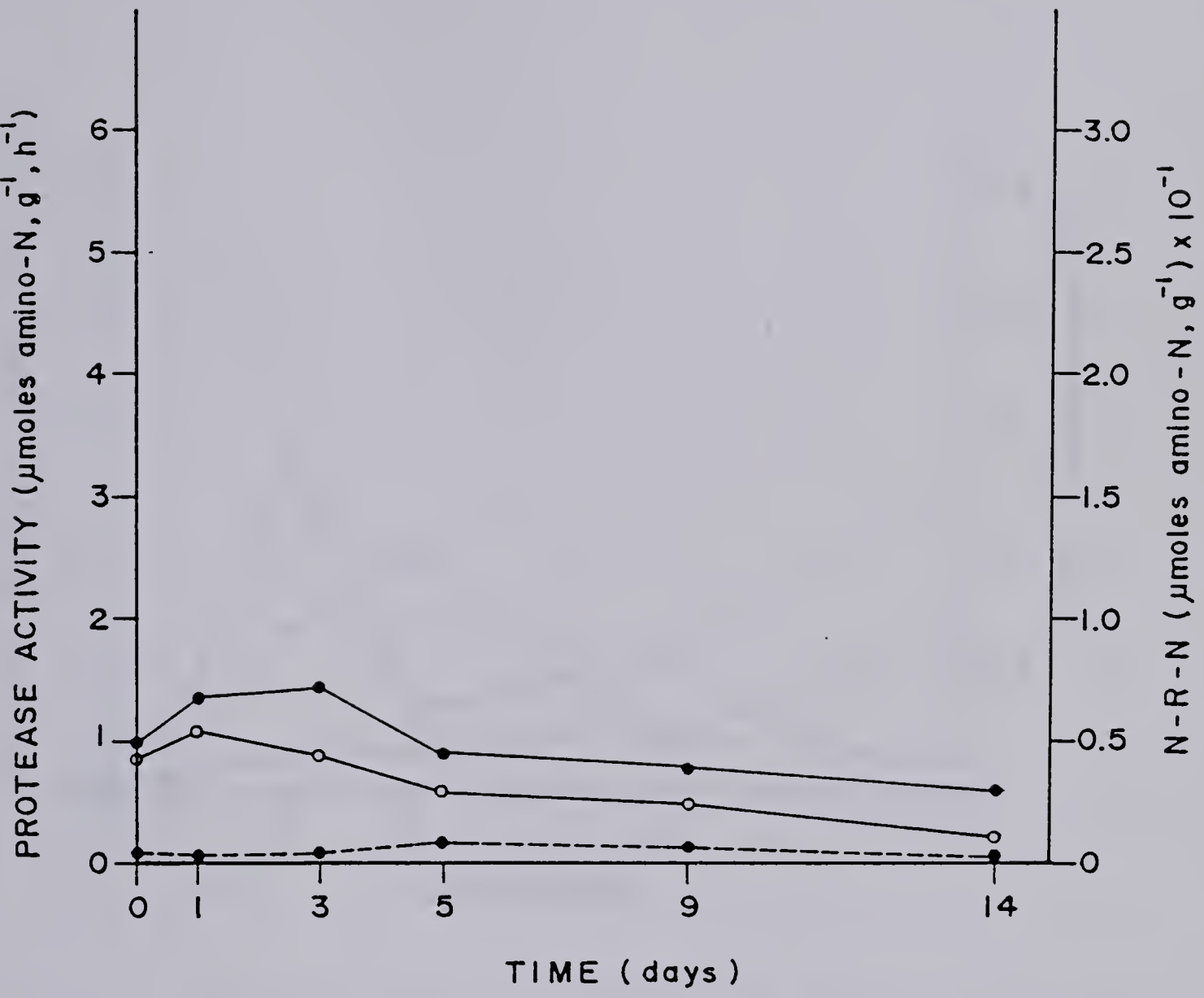

Figure 4.2 Protease activity in a preincubated nonamended Breton soil. (●——●) assay substrate; CBZ-PL. (○——○) assay substrate; casein. (●--●) N-R-N.

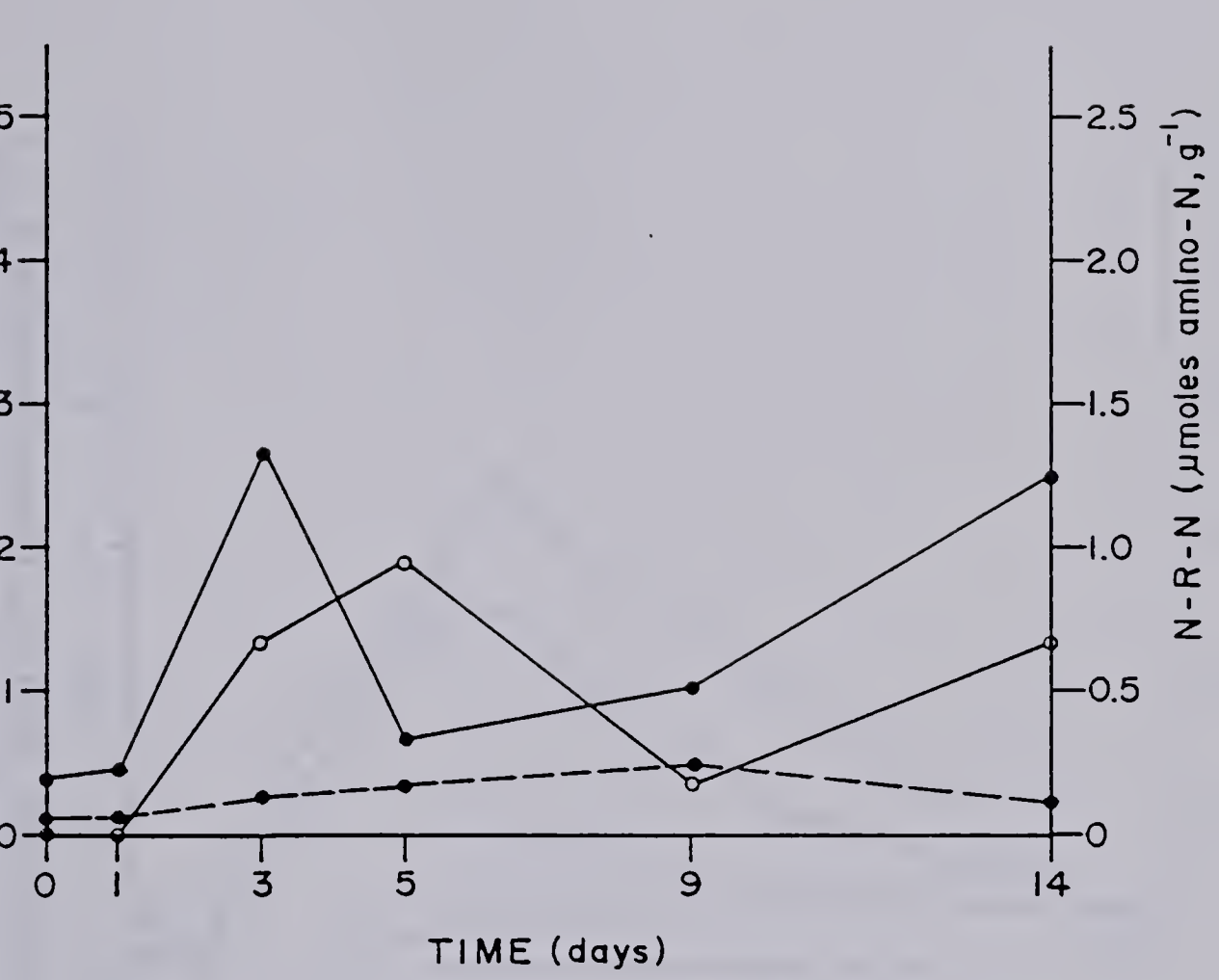

Figure 4.3 Protease activity in a Malmo soil amended with casein, glucose and ammonium sulphate. (●——●) assay substrate; CBZ-PL, (O——O) assay substrate; casein, (●--●) N-R-N.

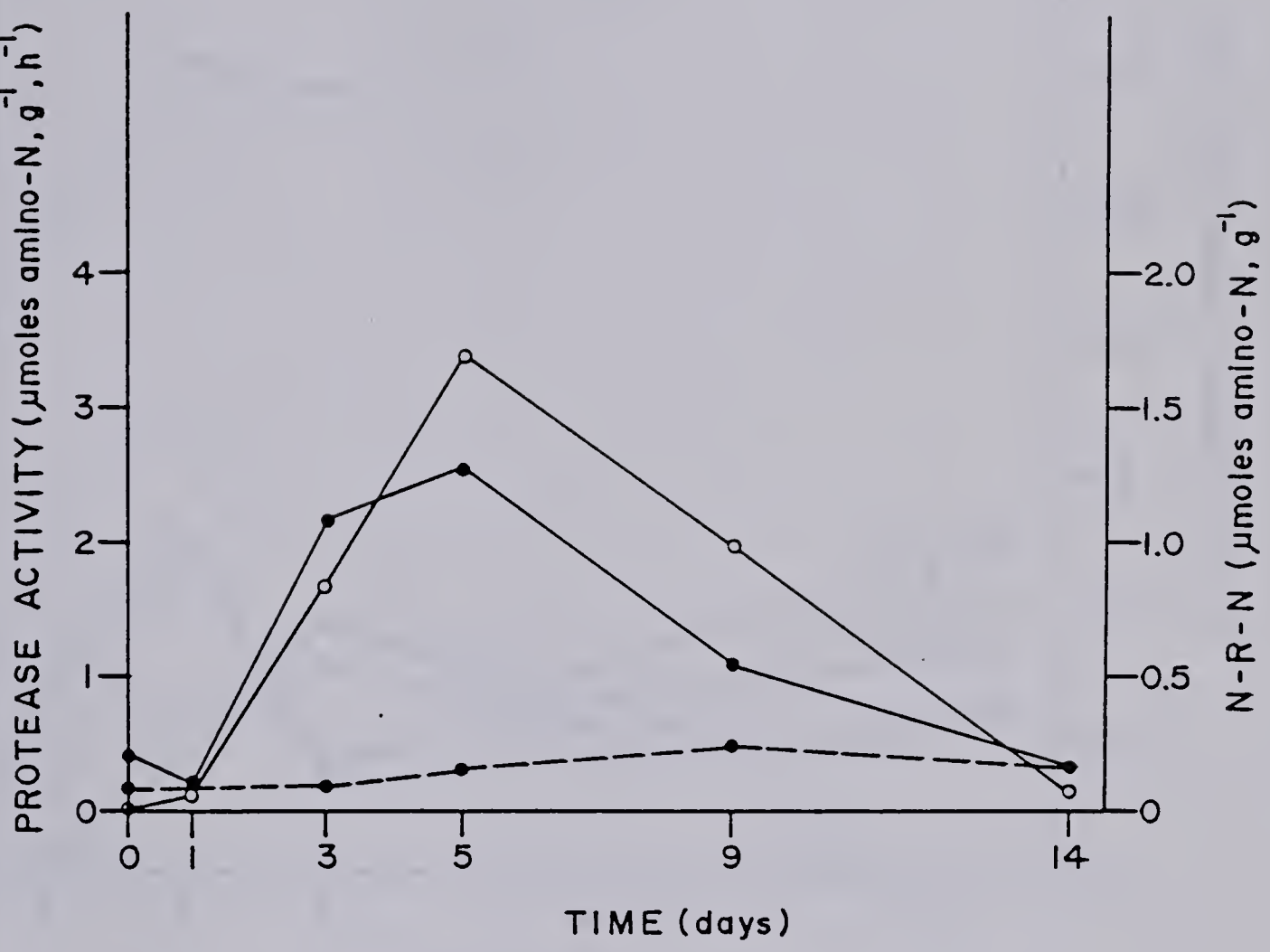

Figure 4.4 Protease activity in a Breton soil amended with casein, glucose and ammonium sulphate with CBZ-PL (●—●) and casein (O—O) as assay substrates. Ninhydrin - reactive - N (●—●).

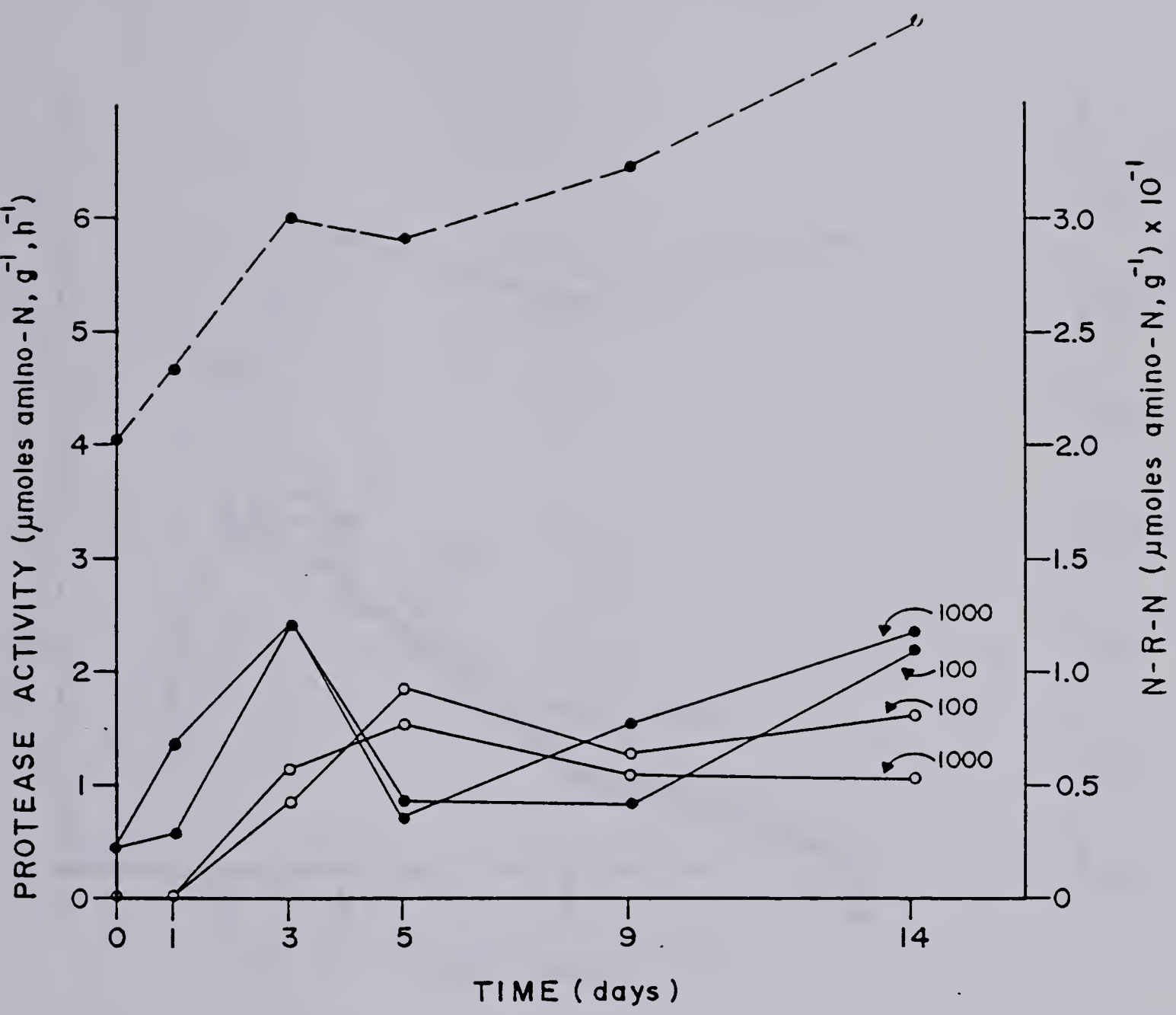

Figure 4.5 Protease activity in a Malmo soil amended with casein and NH_4^+. Assay substrates: (●—●) CBZ-PL, (○—○) casein, (●—●) N-R-N.

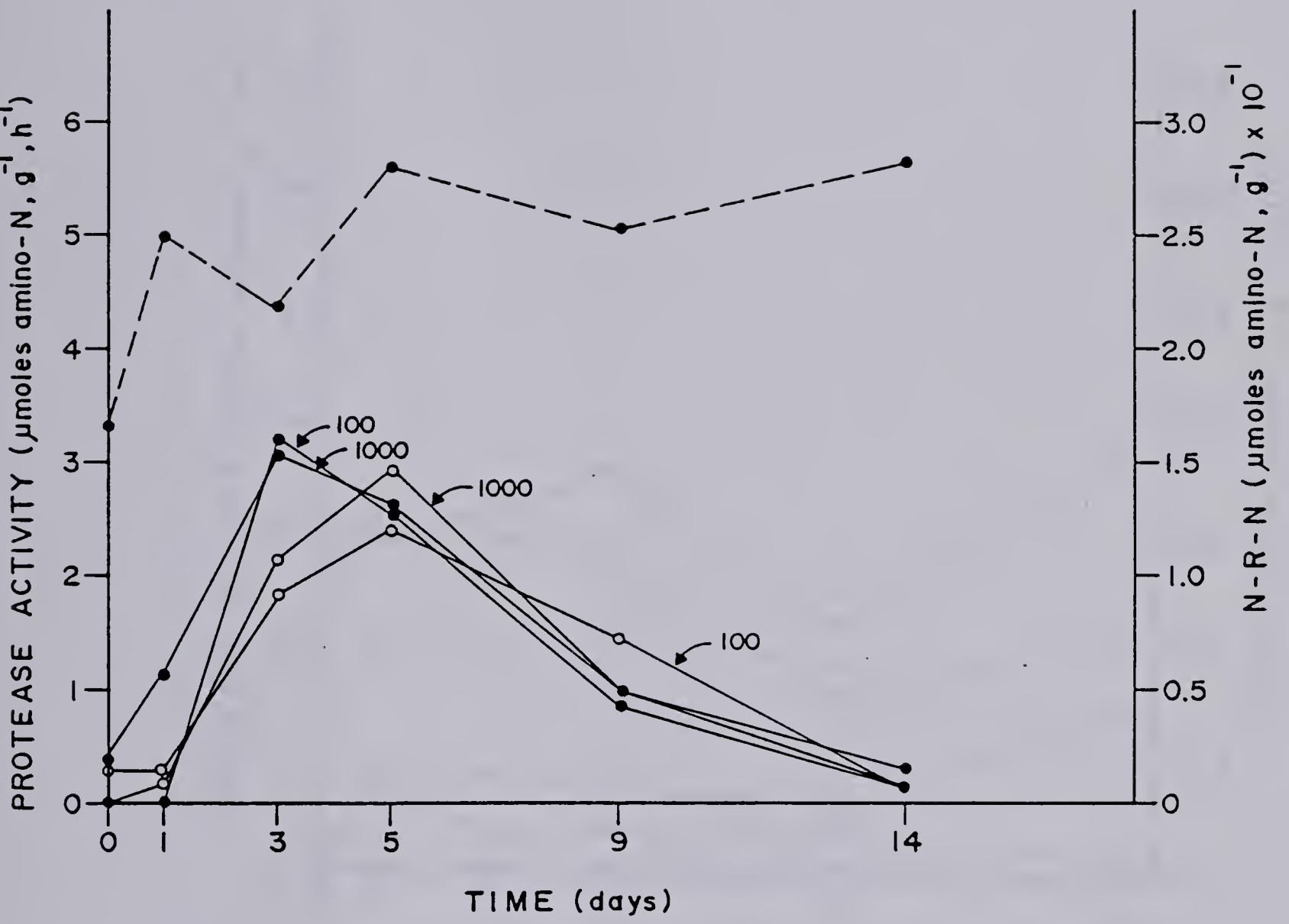

Figure 4.6 Protease activity in a Breton soil amended with casein and NH_4^+. Assay substrates: (●—●) CBZ-PL, (○—○) casein, (●- -●) N-R-N.

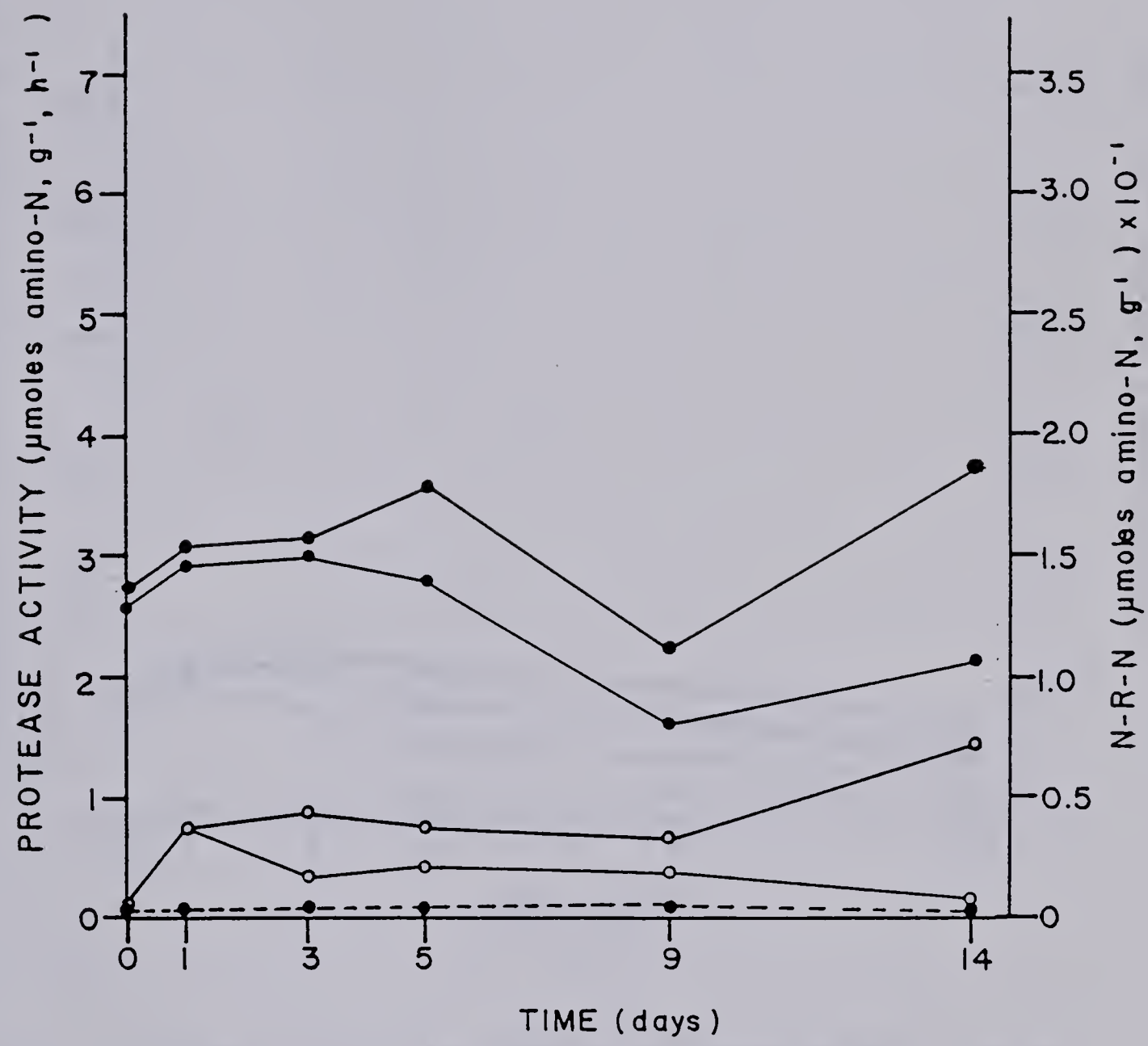

Figure 4.7 Protease activity in a Malmo soil amended with glucose and NH_4^+. Assay substrates: (●——●) CBZ-PL, (○——○) casein, (●- -●) N-R-N.

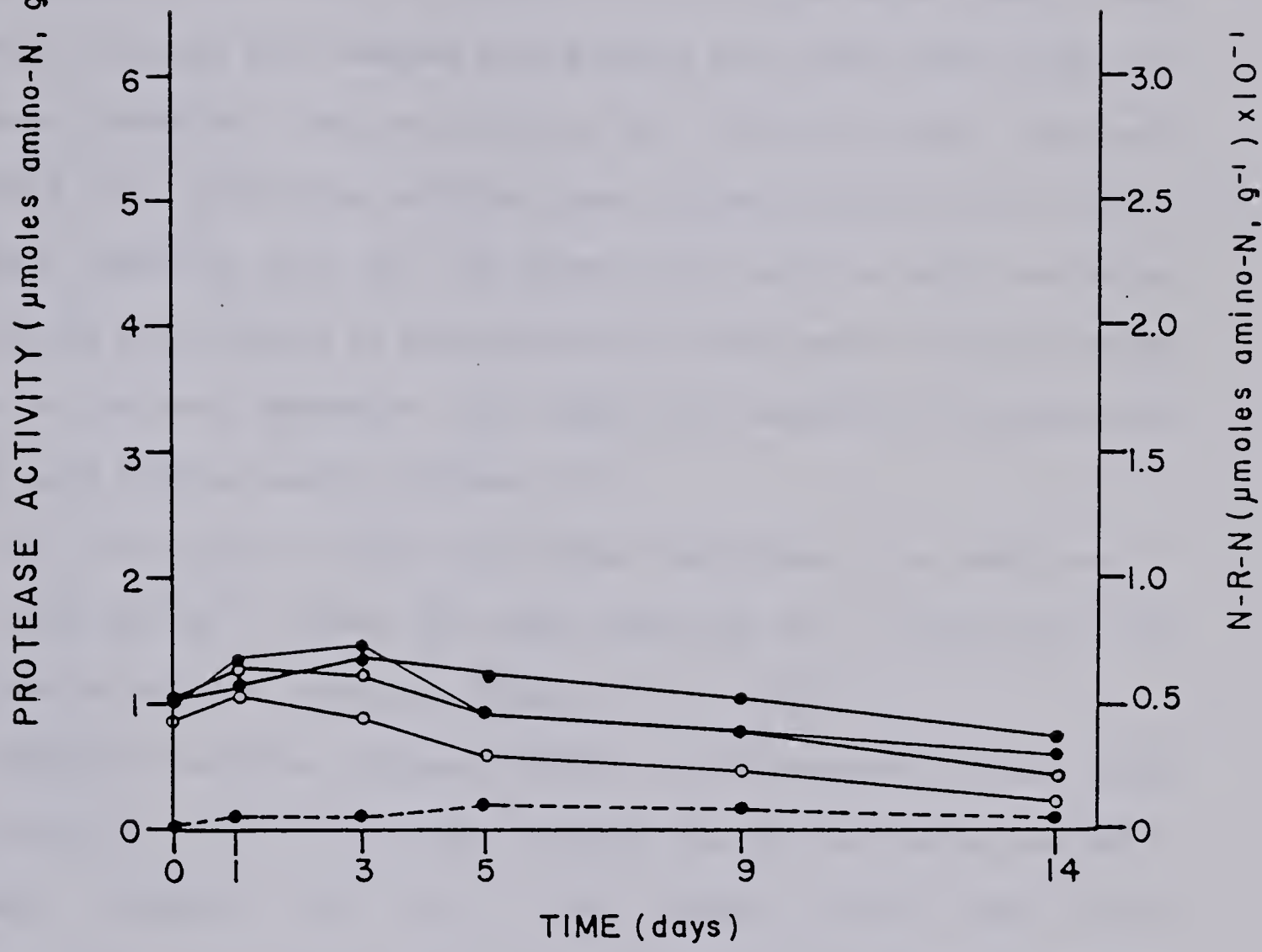

Figure 4.8 Protease activity in a Breton soil amended with glucose and NH_4^+. Assay substrates: (●—●) CBZ-PL, (●—●) casein, (●--●) N-R-N.

Amendment of both soils with casein and glucose gave results that were significantly different from the control soil, and the glucose and NH_4^+ amended soil (Figures 4.9, 4.10).

A higher peak value of protease activity on days 5 and 9 was observed in Breton soil amended with glucose and casein when casein was the assay substrate than was observed for CBZ-PL as assay substrate (Figure 4.10). The Breton soil had lower protease activity with CBZ-PL as assay substrate than did the Malmo soil; but, as with the Malmo soil, a lag was observed in the appearance of the peak value when using casein as the assay substrate. The Malmo soil amended with glucose and casein gave similar results (Figure 4.9).

For both soils and with both assay substrates, the addition of NH_4^+ (1000 mg kg^{-1}) during the assay appeared not to influence the magnitude of protease activity. (Figures 4.11, 4.12)

Ninhydrin Reactive Nitrogen (NRN) levels measured in the same soils show the high levels of NH_4^+ present due to the casein plus NH_4^+ treatment (Figures 4.5, 4.6). The results clearly show that differentiation of protease activity values and NRN values was made possible by the NH_3 removal method. With the exception of those soils amended with NH_4^+, NRN levels measured for other treatments were consistently low. (Figures 4.1, 4.2, 4.3, 4.4)

Series II (no preincubation)

Nonamended soils assayed with and without substrate, consistently indicate a base level measurement of 0.5 - 1.5 mg amino -N kg^{-1} (Figure 4.13). The results for the Malmo soil were consistently higher, approximately 1 mg amino-N kg^{-1} than for the Breton soil.

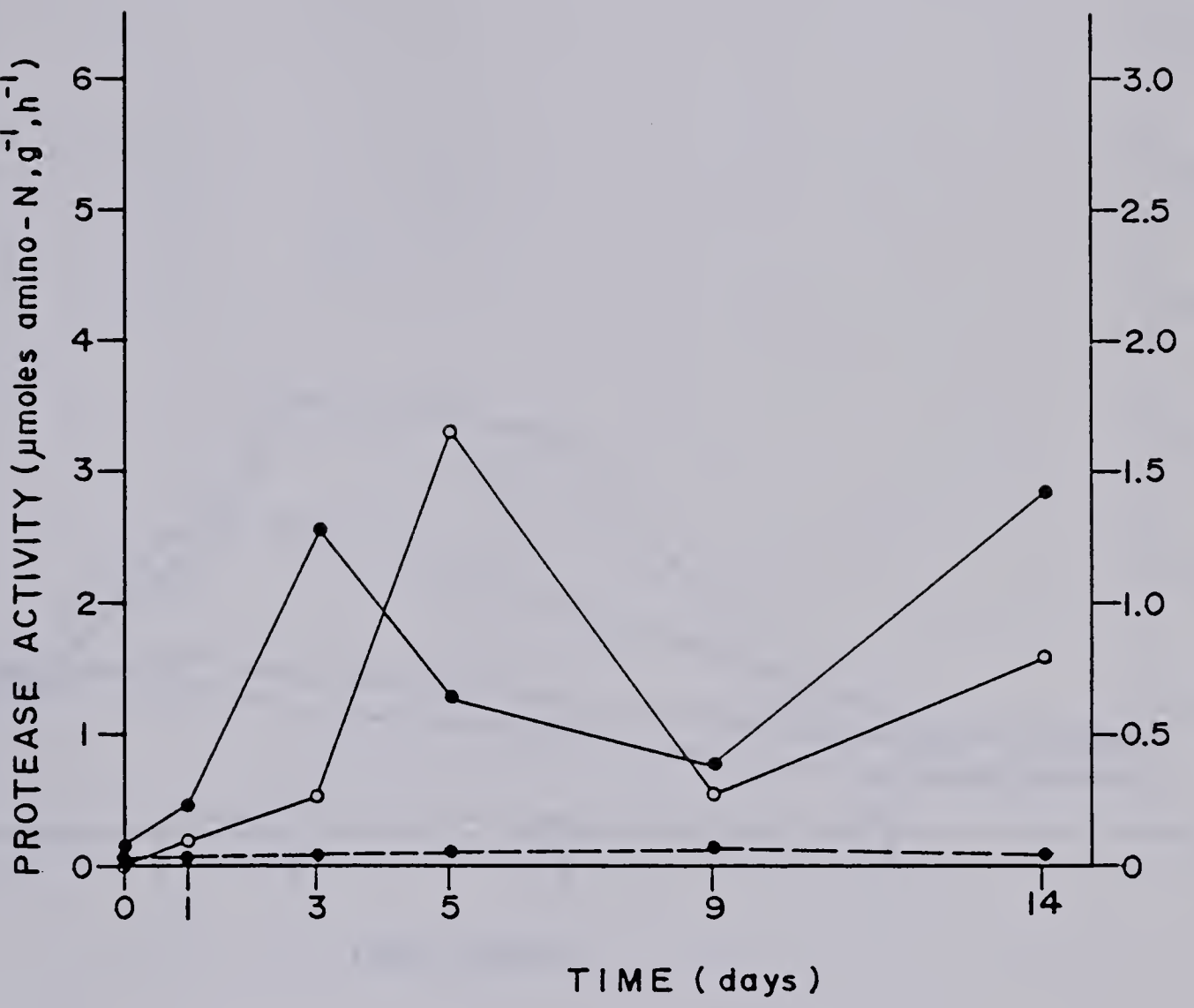

Figure 4.9 Protease activity in a Malmo soil amended with glucose and casein. Assay substrates: (●—●) CBZ-PL, (○—○) casein, (●--●) N-R-N.

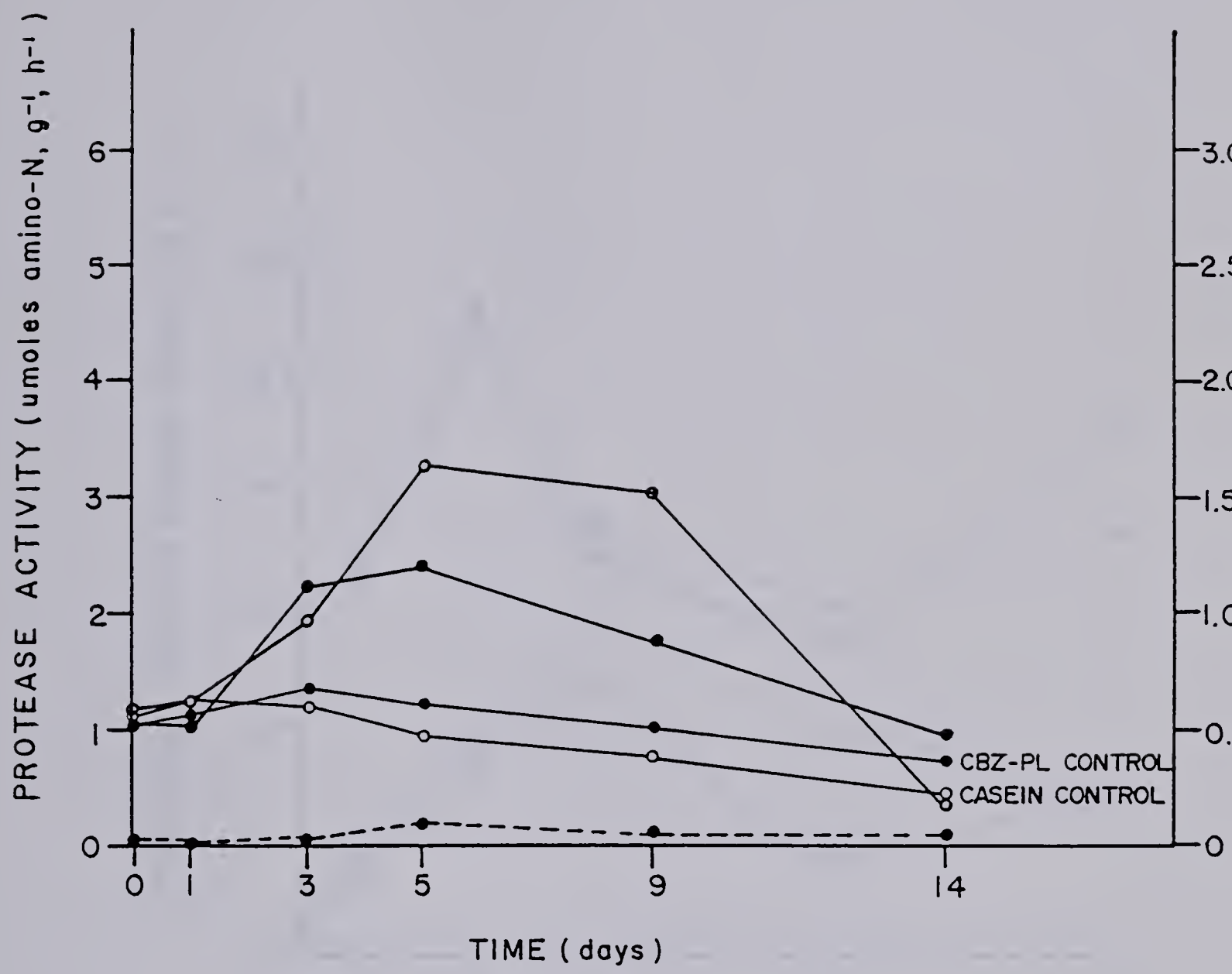

Figure 4.10 Protease activity in a Breton soil amended with glucose and casein. Assay substrates: (●——●) CBZ-PL, (○——○) casein (●- -●) N-R-N.

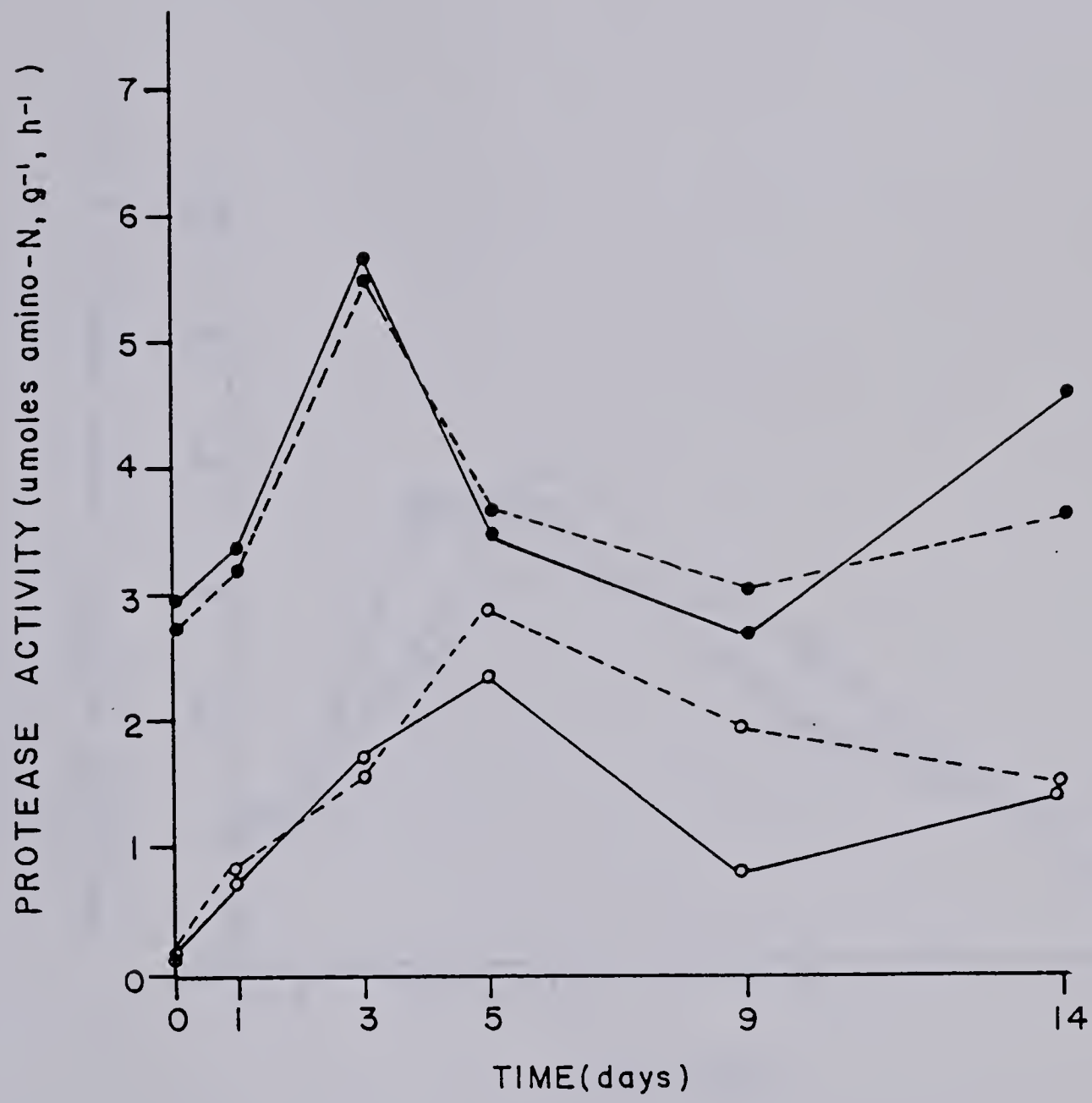

Figure 4.11 Protease activity in a Malmo soil amended with casein and assayed in the presence and absence of NH_4^+ (at 1000 mg NH_4-N·kg^{-1} soil). Assay substrates: (●——●) CBZ-PL, (○——○) casein. Amendment with NH_4^+ (- - - -), no added NH_4^+ (————).

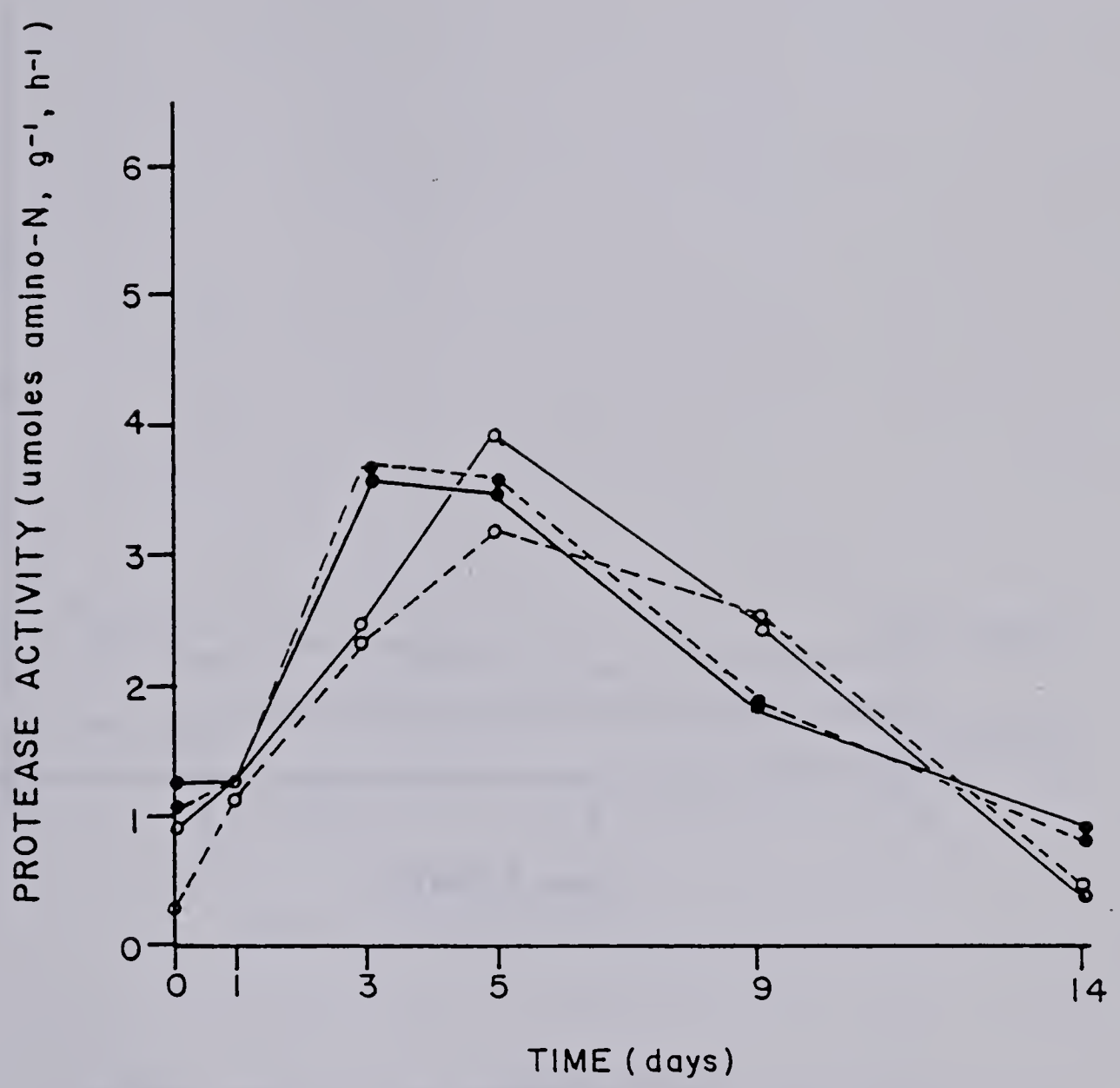

Figure 4.12 Protease activity in a Breton soil amended with casein and assayed in the presence and absence of NH_4^+ (at 1000 ug NH_4-N, g^{-1} soil). Assay substrates: (●—●) CBZ-PL, (O—O) casein. Amendment with NH_4^+ (– – – –), no added NH_4^+ (———).

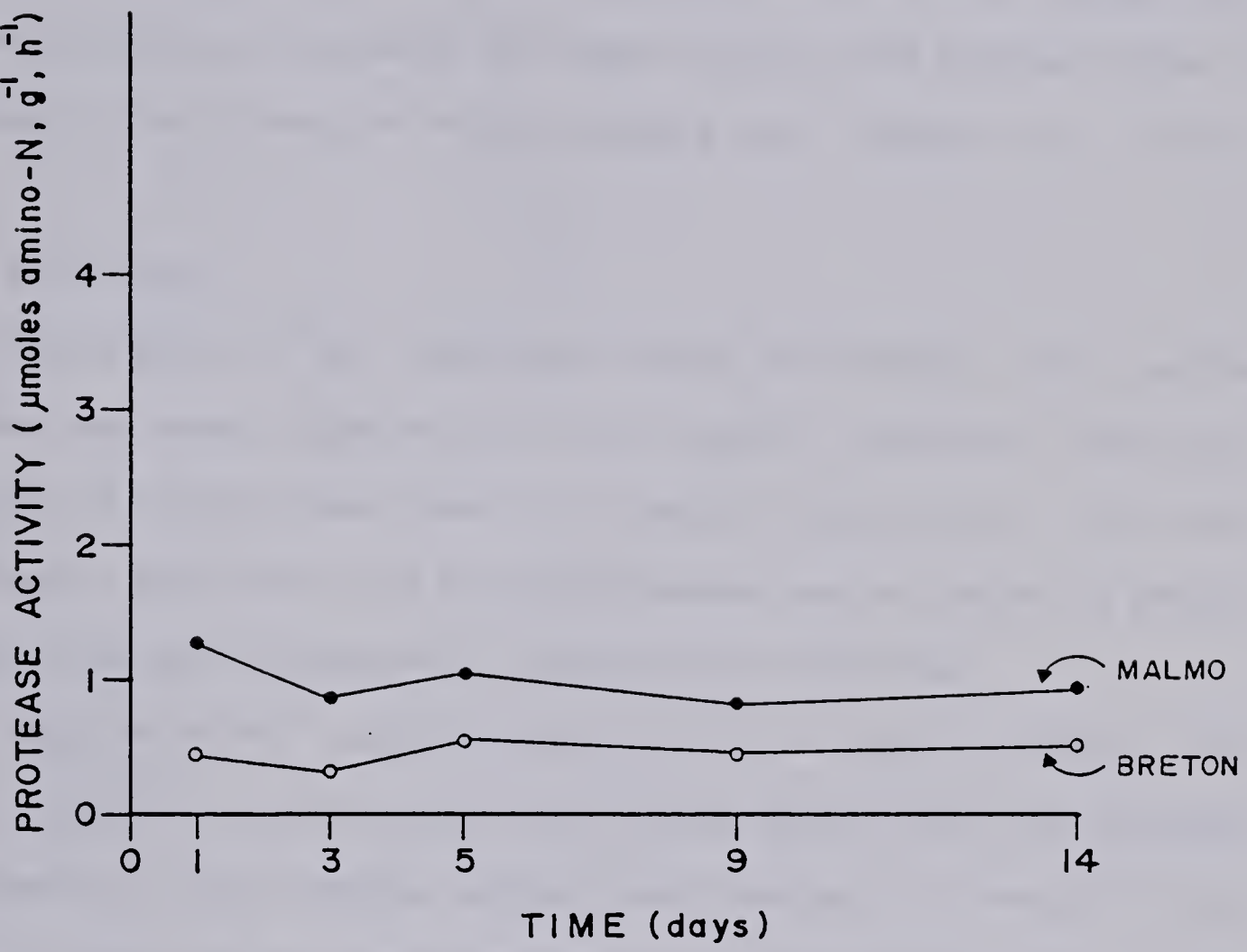

Figure 4.13 Protease activity in unamended Malmo and Breton soil during incubation.

Statistical analysis of the data indicated a significant difference between the treatment means for glucose +NO_3^--N (with C @ 3,000 mg kg^{-1}), the means of the other two treatments, and the control ($p=0.01$), in both soils over the 14 day incubation period (appendix). The differences in the means were small as were the maximal values of protease activity recorded at any sampling time (Figures 4.14, 4.15).

4.4 Discussion

The results of the experiments using preincubated soils confirm earlier experiments (Section 3.1) that showed a consistent significant increase in activity when casein is incubated with the soil. All other amendments, which were of a non-proteinaceous nature, failed to produce results that were significantly different from controls.

Addition of NH_4^+ either incubated with the soil or added to the assay mixture at concentrations up to 1000 mg kg^{-1} did not influence the results. This confirms earlier work (Section 3.1) where NH_4^+ was added to the soil at concentrations up to 400 mg kg^{-1}. The results also show clearly the efficiency of the NH_3 removal method used throughout this study.

The results obtained were consistently different between the two soils with respect to maximal activity levels and apparent substrate specificity. In the Malmo soil, CBZ-PL as substrate yielded higher measures of activity than did casein. Activity in the Malmo soil exceeded that in the Breton, when CBZ-PL was used as an assay substrate. In the Breton soil the highest increase in protease activity was observed using casein as the assay substrate, and was consistently higher than that recorded for the Malmo soil using the

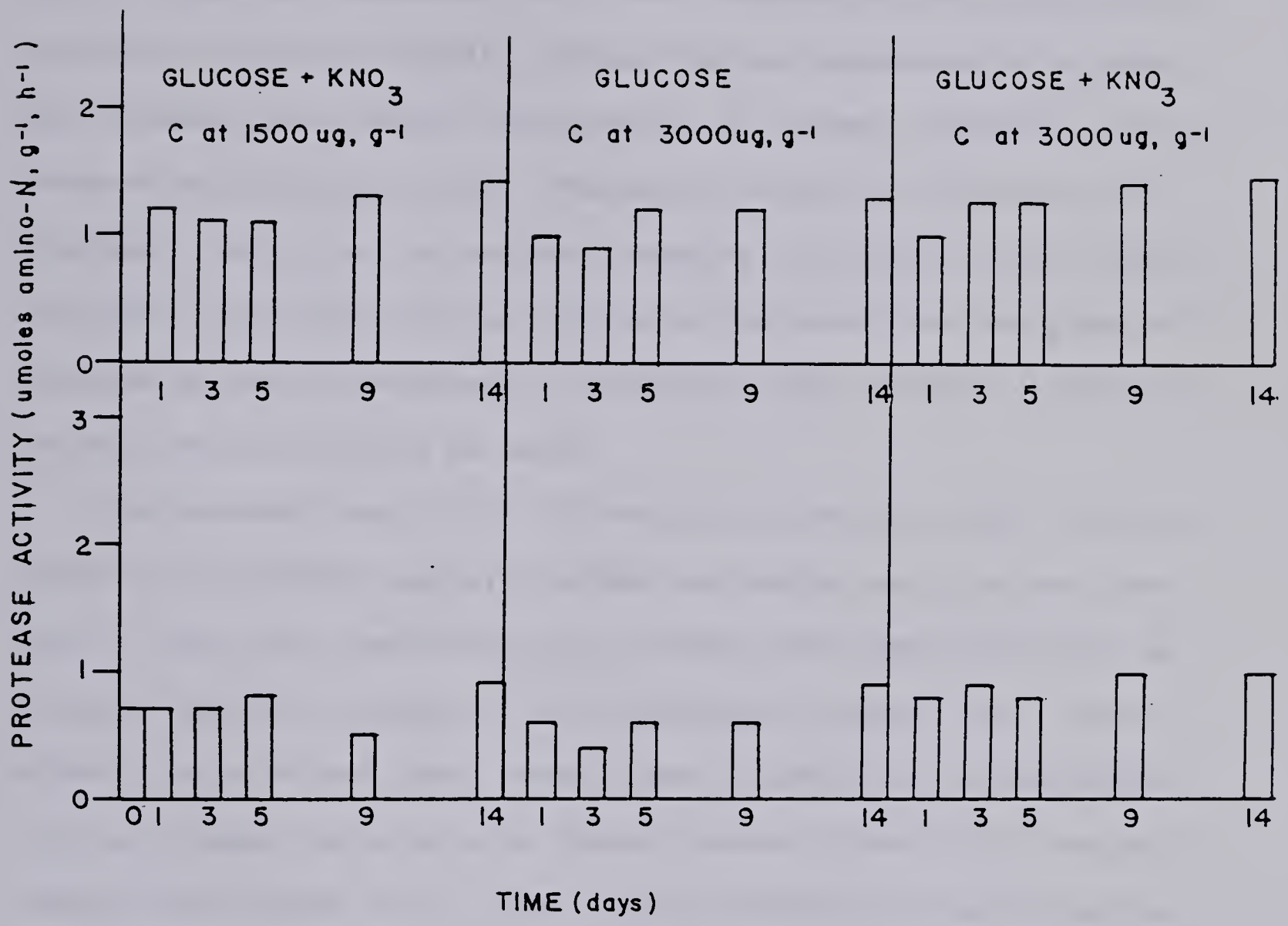

Figure 4.15 Protease activity in a Malmo soil amended with glucose and KNO_3. No prior preincubation.

Figure 4.14 Net Protease activity in a Breton soil amended with glucose and KNO_3. No preincubation.

same substrate. These results would suggest that there are differences in substrate specificities of the protease enzyme "pools" in the two soils. This is consistent with the observation of a 2-3 fold difference in maximal activity between the two substrates in the Malmo soil whereas much smaller differences in maximal activities were observed in the Breton soil. Further, for both soils there was a consistent delay in the maximal response to casein as an assay substrate, which supports the hypothesis that more than one group of protease enzymes or organisms is involved in the increase of protease activity observed for the two soils.

The apparent specificity differences in the two soils and the differences in their respective maximal activities would indicate that one or more soil characteristics influence the type and amount of protease activity present. An association between the protease activity of soils and their chemical and or physical characteristies has been sought but with only limited success (Ladd 1972; Ross and McNeilly 1975; Ross 1977). Some of their results indicate that the protease activity of some soils can be positively and significantly correlated with the amounts of mineral nitrogen produced during incubation of the soil. With other soils however, positive correlations were not found and were mainly non-significant (Ross and McNeilly 1975). It is likely that the lack of consistent correlations of protease activity with the physical and or chemical characteristics of soil is due, at least in part, to the existence of many different proteases in soil (Ladd and Butler 1972 Kiss et al 1975). Further, the differences due to structural constraints in the susceptibility to degradation of native proteinaceous compounds added to or retained in

different soils may influence the type and therefore the measured activity of proteases when examined under laboratory conditions using other proteins, or parts thereof, as substrates. The substrates used in this study are readily hydrolysed and may not provide a precise measure of native proteolytic potential. However, the trend is clear. Soils differ widely in their measureable proteolytic activity and in their response to added proteins. Such a trend, confirmed by the results of this study, gives evidence of the genetic diversity of soil microbial populations and of modes of enzyme stabilization and activity in soil.

When soils are air dried and stored some enzymes are denatured resulting in an initial lowering of total enzyme activity compared with fresh field moist samples (Ambroz in Ross 1977; Speir and Ross 1975). Air drying also results in an increase in nitrogen mineralization following re-wetting of the soil. Due to instability of the enzyme pool and the flush of mineral nitrogen following re-wetting the soil, Ladd and Butler (1972) suggested that preincubation of soil was desireable so that variability in protease activity measurements resulting from these factors was minimized. Ladd and Paul (1973) in measuring various enzyme activities in non-preincubated soils, during $^{15}NO_3^-$ immobilization experiments with glucose and NO_3^--N, observed an increase in protease activity towards the substrates casein and CBZ-PL that was similar to the results of this study using preincubated soils with casein amendment. We have been unsuccessful in demonstrating an increase in measured protease activity of the magnitude they reported using glucose and NO_3^--N, either in combination or alone, in non-preincubated soils. This study coupled with the

earlier experiments shows that in the Breton and Malmo soils protease activity levels are only weakly, if at all, affected by the addition of glucose $+NO_3$-N amendments. These results support the hypothesis that soil proteases are inducible.

In formulating an hypothesis about protease control in soil, information is required about known control mechanisms. Intra- or extracellular degradative enzymes which supply metabolites to cells are called Class I enzymes (Mandelstam and McQuillen 1976). They can be either constitutive or inducible and may be subject to controls. Biological conservation of materials permits growth and differentiation of cells to be coordinated with the requirements and availability of metabolites. Regulation of constitutive enzymes is by specific inhibition of the enzyme activity whereas inducible enzymes are also subject to repression of synthesis (Mandelstam and McQuillen 1976). Derepression of inducible enzyme synthesis requires the interaction of an inducer molecule or molecules, which may be the substrate itself, a sub-component of same, or even another type of molecule. An example of the latter is a permease, required for transport of the substrate in the B-galactosidase system in E. coli (Mandelstam and McQuillen 1976). Further, some genetic mutant strains of bacteria lack the ability to produce a repressor molecule and consequently the cell produces enzyme in a constitutive manner, whether the inducer molecule is present or not (Mandelstam and McQuillen 1976; Davis et al 1973).

An additonal form of enzyme regulation demonstrated for some enzymes is catabolite repression or the "glucose effect" (Magasanik 1961). In its simplest terms, a product of the enzymic reaction

interacts with genes coding for synthesis of that particular enzyme and no new enzyme is produced. Catabolite repression does not influence the activity of existing enzymes.

In light of the possibilities for regulation of enzyme activity and/or synthesis that have been demonstrated in pure cultures it is probable that in mixed populations of microorganisms in soil, one or more of the aforementioned regulatory mechanisms may be operating for a given set of soil physical and chemical conditions.

4.5 Summary

Both soils used in this study showed reproducible increases in measured protease activity when casein was incubated with the soil. Results obtained with all other amendments, and most specifically glucose, were much lower than obtained with casein.

Protease activity was consistently different both in maximal values reached and apparent substrate preference for the two soils used. The results for the Malmo soil showed consistently higher maximal values of protease activity with CBZ-PL, and also showed consistent differences in maximal values for the two assay substrates used. The results for the Breton soil were consistently higher when casein was the assay substrate and showed smaller differences between the two substrates used than was observed in the Malmo soil. Measured protease activity was highest in the Malmo soil.

In both soils the presence of NH_4^+ at a concentration of 1000 mg kg^{-1}, either incubated with the soil, or in the reaction mixture for the protease assay, failed to significantly alter, positively or negatively, protease activity resulting from casein amendment

of the soil. Use of air dried soils not preincubated before use, failed to overcome the difference between glucose and casein amendments.

5. Control Mechanisms

5.1 Introduction

> It is well known that perpetuation of life on our planet is conditioned by the mineralizing action of soil and water microorganisms on the plant and animal residues. It is also well known that the mineralizing action of micro-organisms is inseparably related to the activity of enzymes.
>
> (Kiss et al, 1975)

Information about the activities of soil enzymes and changes in the activity of those enzymes has provided information that has been variously used to describe and characterize the role of soils in the global cycling of matter. This information is vital to our understanding of soil processes and ultimately of how man may influence these processes. Much of the information about soil enzymes relates to gross measurements of activity and changes in that activity under controlled conditions.

Experiments described in sections 3 and 4 demonstrated a reproducible increase in protease activity in Breton and Malmo soils when casein was added to, and incubated with, the soils. Additions of glucose during the incubations, and of NH_4^+ either during the incubation or during the protease assay had little effect on the increase in activity associated with casein amendment. Further, incubation with glucose alone or in combination with either NH_4^+ or NO_3^- generated much less activity than did addition of casein regardless of whether the soils were preincubated or were dry prior to adding the amendments.

Some explanation for the stimulatory effect of casein and the apparent insensitivity of protease production to glucose is needed, because these results conflict with those of Ladd and Paul (1973) who reported increases in protease activity of several fold during the 14

14 day incubation period following glucose addition.

Very little information is available concerning the regulation of enzyme production and activities in soil. Traditionally reference is made to experiments carried out in solutions using pure cultures for information about the possible forms of enzyme regulation that may be operating in soils. This approach is reasonable but poorly justified. It is reasonable in light of the similarity of organisms studied and their cellular requirements. It is poorly justified when one compares the environments of the cells and enzymes under study in the two separate systems.

Catabolite repression and end product inhibition of enzyme synthesis or activity have been demonstrated in pure cultures using several microbial species (Sec. 2.1 Literature Review). Experiments described in this study were designed to investigate the possibility that these regulatory processes can be demonstrated in soils.

The primary hypothesis was that regulation of protease synthesis and/or activity, in soils, was consistent with regulatory processes demonstrated in pure cultures.

To examine the possibility of a stimulatory effect on protease synthesis arising from degradative products of proteolysis, incubation experiments were performed separately using three amino acids (a neutral, a basic, and an acidic amino acid) and two di-peptides along with a carbon source (glucose) as amendments to the Breton and Malmo soils. Protease activity measurements were made in the presence and absence of assay substrate to eliminate analytical artifacts due to the amendments.

A further set of experiments was carried out using the Malmo soil to test the hypothesis that repression of protease synthesis as described by Bromke and Hammel (1979) was significant. Casein increases protease activity, and rifampicin, a transcriptional inhibitor prevents transcription of the genetic code required for translation in the synthesis of some proteins, among them exoproteases (Bromke and Hammel 1979). Therefore rifampicin was added to the incubated Malmo soil, with and without casein, to look for evidence of repression of enzyme synthesis at the transcriptional level.

To examine the possible role of catabolite repression, as it may be involved in protease synthesis or activity expression, experiments were performed utilizing cyclic adenosine mono-phosphate (c-AMP) and dibutyryl c-AMP in soils amended separately with casein, glucose and NO_3^- and glucose and NH_4^+. The experiments using soils amended with glucose and NO_3^- or glucose and NH_4^+ were designed to further document the differences in magnitude of results between earlier experiments (sections 3 and 4) and those of Ladd and Paul (1973). Dibutyryl c-AMP was employed because it has been reported that this derivative was more permeable and resistant to extracellular degradation (Kankel and Hirtz in Bromke and Hammel, 1979).

5.2 Materials and Methods

Soils The Breton and Malmo soil samples used were from the same source as previously described (section 4.2). All soils were preincubated at 25% H_2O (w/w) content for a minimum 10 day period at room temperature (18°) prior to experimental start up. Maximum time of preincubation was 14 days.

Soil Amendments

Unless otherwise indicated amino acids and di-peptides were added as amendments on the basis of their nitrogen content at the rate of 100 mgN kg^{-1}. Glucose was added at the rate of 1500 mg C kg^{-1}.

In experiments using c-AMP, dibutyryl c-AMP, and/or rifampicin, amendments were added at the following rates.

Casein @ 1500 mg C kg^{-1}

Glucose @ 1500 mg C kg^{-1}

NO_3^- @ 100 mg N kg^{-1}

NH_4^+ @ 100 mg N kg^{-1}

c-AMP @ 5 umoles ml^{-1} water in the soil

dibutyryl c-AMP @ 5 umoles ml^{-1} water in the soil

rifampicin @ 3 umoles ml^{-1} water in the soil

Experimental Treatments:

Series I: Objective: To examine the possible stimulation of protease synthesis arising from degradative products of proteolysis. Treatments as follows:

1) control soil (no amendments)
2) Leucine and glucose
3) Lysine and glucose
4) Phenylalanine and glucose
5) glycylglycine and glucose
6) glycyl-L-leucine and glucose

All treatments were assayed in triplicate with and without assay substrate (CBZ-PL).

Series II: Objective: To test the hypothesis that control of protease synthesis, or activity, by catabolite repression can be derepressed in

the soil used in this study; further, to test the hypothesis that transcription of the code for synthesis of protease activity may be repressed by rifampicin resulting in no net synthesis upon stimulation with casein.

Treatments were as follows:

1) control soil
2) casein
3) casein and rifampicin
4) rifampicin (control)
5) casein and rifampicin (rifampicin added on day 3)
6) glucose and KNO_3
7) glucose and KNO_3 and c-AMP
8) c-AMP control
9) glucose and NH_4^+
10) glucose and NH_4^+ and c-AMP
11) glucose and NO_3^--N and di-but c-AMP
12) glucose and NH_4^+-N and di-but c-AMP

All treatments were assayed in triplicate with and with-out substrate (CBZ-PL).

All experimental treatments when assayed for protease activity were subjected to free-NH_3 removal by the method previously described (Sec. 3 Materials and methods).

Chemicals:

All chemicals used were enzyme assay grade with the exception of buffers and pH adjusting solutions which were reagent grade. Amino acids and di-peptides were obtained from the Sigma Chemical Company and were all of the levo (L) configuration.

5.3 Results

Series I

Amendment of both soils with amino acids and di-peptides resulted in initially high readings. (Figures 5.1,5.2,5.3 and 5.4). The use of substrate free blanks showed that these initially high levels were due to the amendments. By day 5 and thereafter, protease activity levels in treated soils were not significantly different from control soils ($p=0.01$).

Figures 5.5,5.6,5.7, and 5.8 depict the results of the experiment in terms of net protease activity, after both the control and assay blank have been subtracted from the initial observed protease activity values. In both soils and for all treatments, maximum net protease activity measurements recorded were less than 0.8 umoles amino -N g^{-1} h^{-1}.

Series II

Protease activity in the control treatment moistened to 30% H_2O was consistent with results previously obtained, was independent of time and varied between 0.7 and 1.2 mg amino -N released g^{-1} h^{-1} (Figure 5.9a).

Amendment of the Malmo soil with casein gave results (Figure 5.9b) consistent with those of previous experiments (Sec. 3 and 4) in which protease activity increased to a maximum value on day 3 of the incubation period and declined rapidly to the level of the control soil by day 5.

When rifampicin was included along with casein there was a broadening of the activity peak (Figure 5.9c). Results of the rifampicin control (Figure 5.9d) indicated a small contribution to

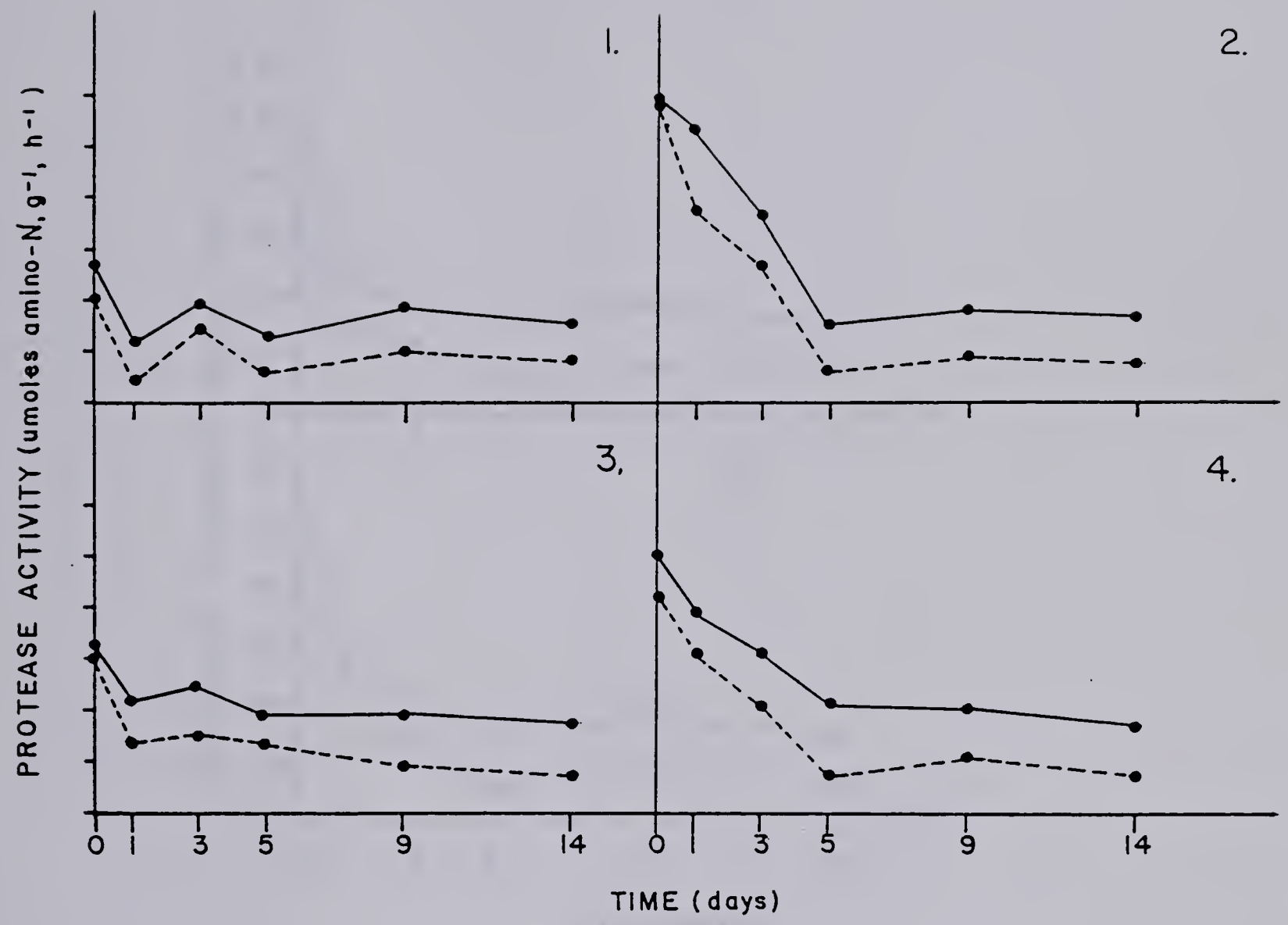

Figure 5.1 Protease activity in a Breton Soil nonamended (control) and amended with amino acids:

1. control
2. leucine and glucose
3. lysine and glucose
4. phenylalanine and glucose

(———) + substrate, (- - - -) - substrate.

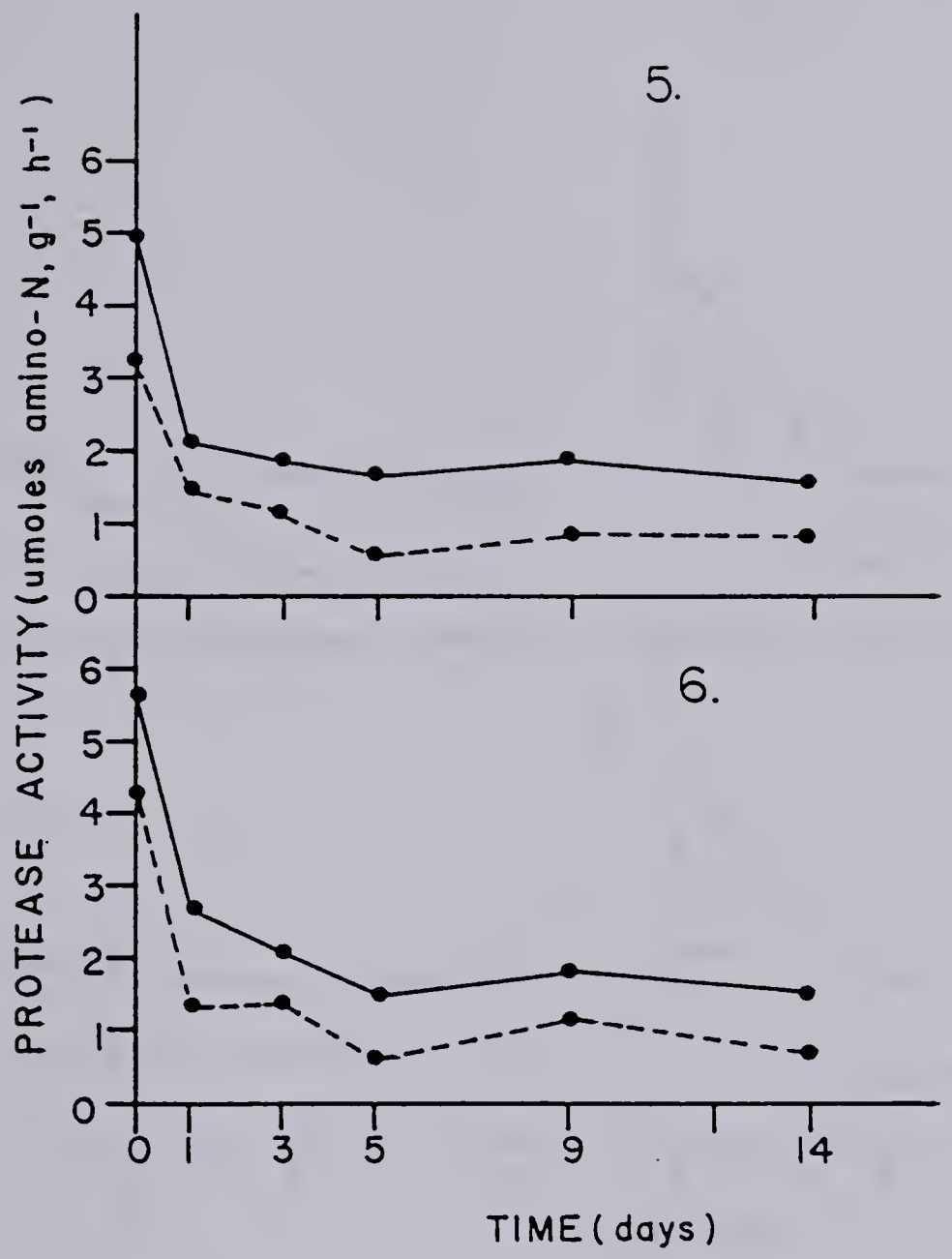

Figure 5.2 Protease activity in a Breton soil amended with dipeptides:

5. glycyl-L-leucine and glucose
6. glycylglycine and glucose.

(———) + substrate, (— — —) - substrate

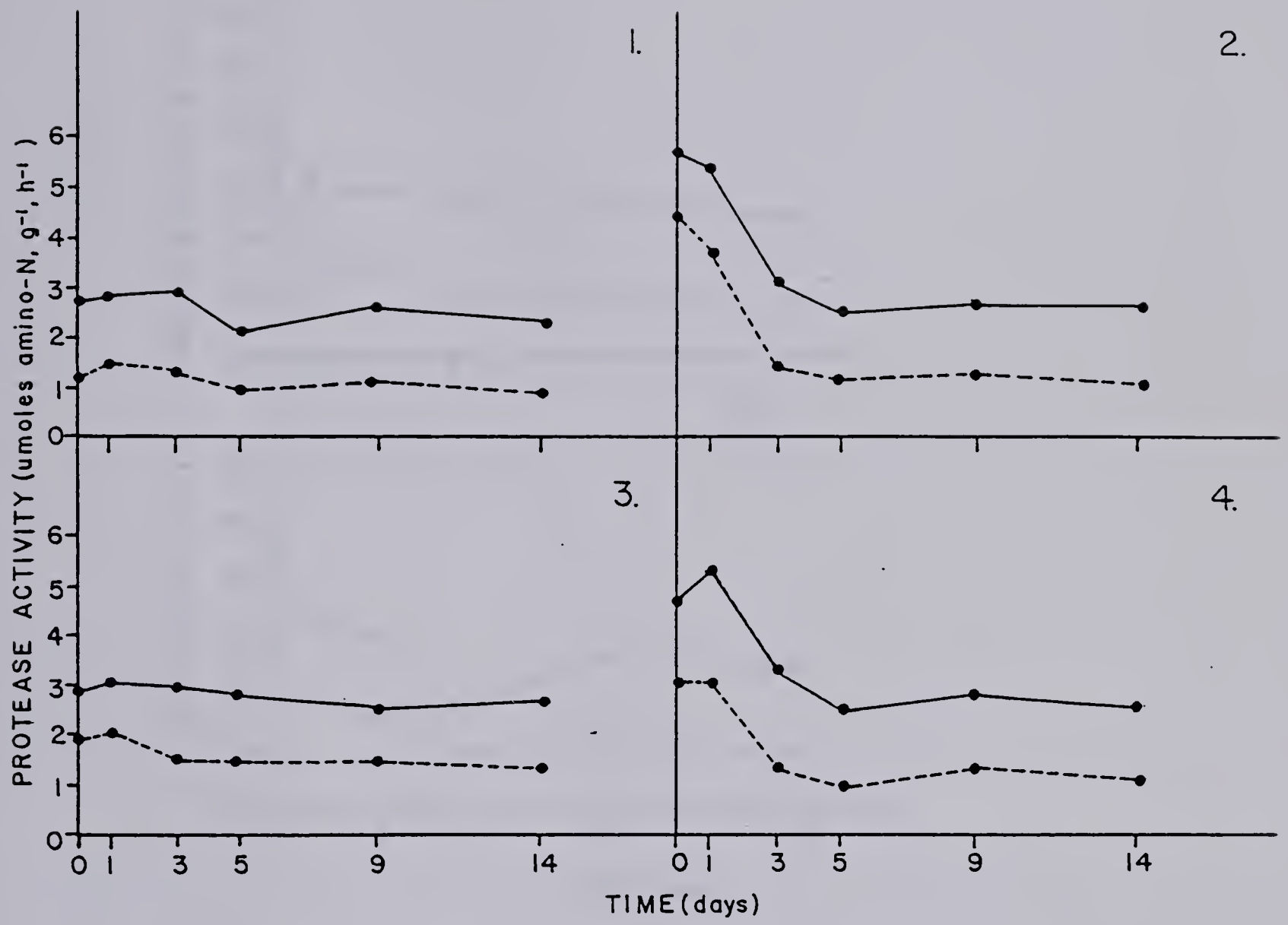

Figure 5.3 Protease activity in a Malmo soil unamended (control) and amended with amino acids:

1. control
2. leucine and glucose
3. lysine and glucose
4. phenylalanine and glucose.

(———) + substrate, (- - - -) - substrate

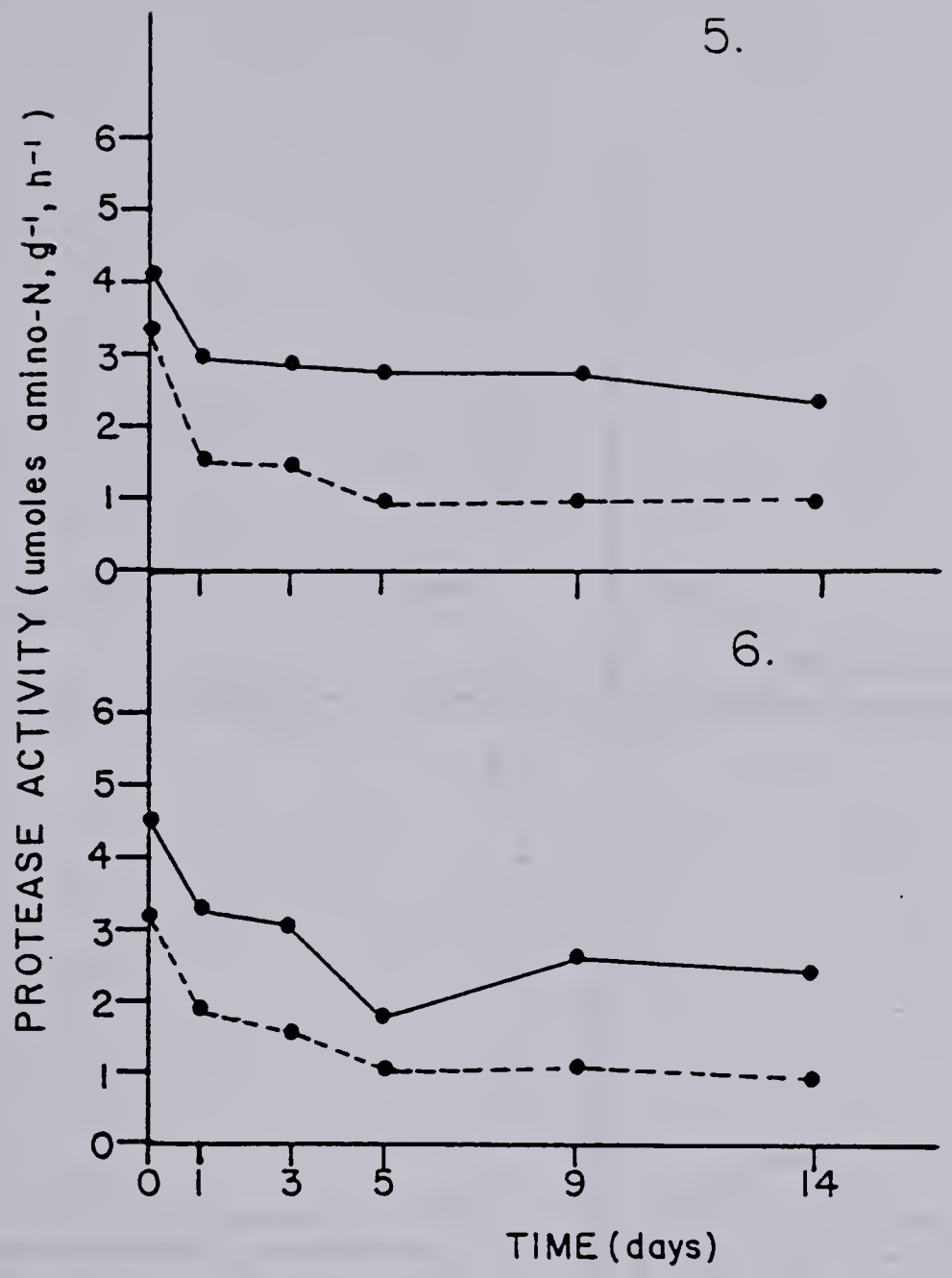

Figure 5.4 Protease activity in a Malmo soil amended with dipeptides:
5. glycyl-L-leucine and glucose
6. glycylglycine and glucose.
(———) + substrate, (— — —) - substrate

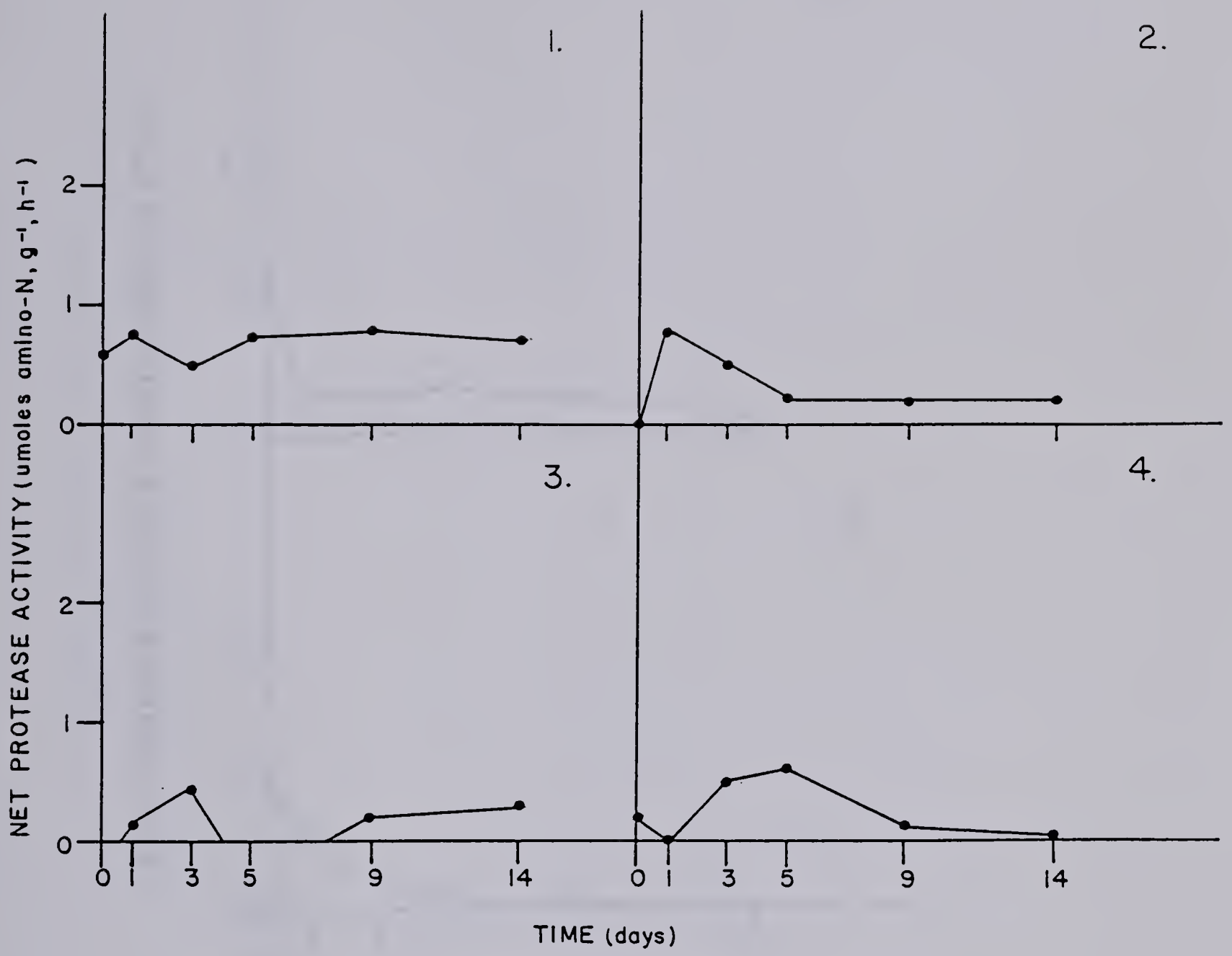

Figure 5.5 Net protease activity in Breton soil unamended control and amended with amino acids:

1. control
2. leucine and glucose
3. lysine and glucose
4. phenylalanine and glucose.

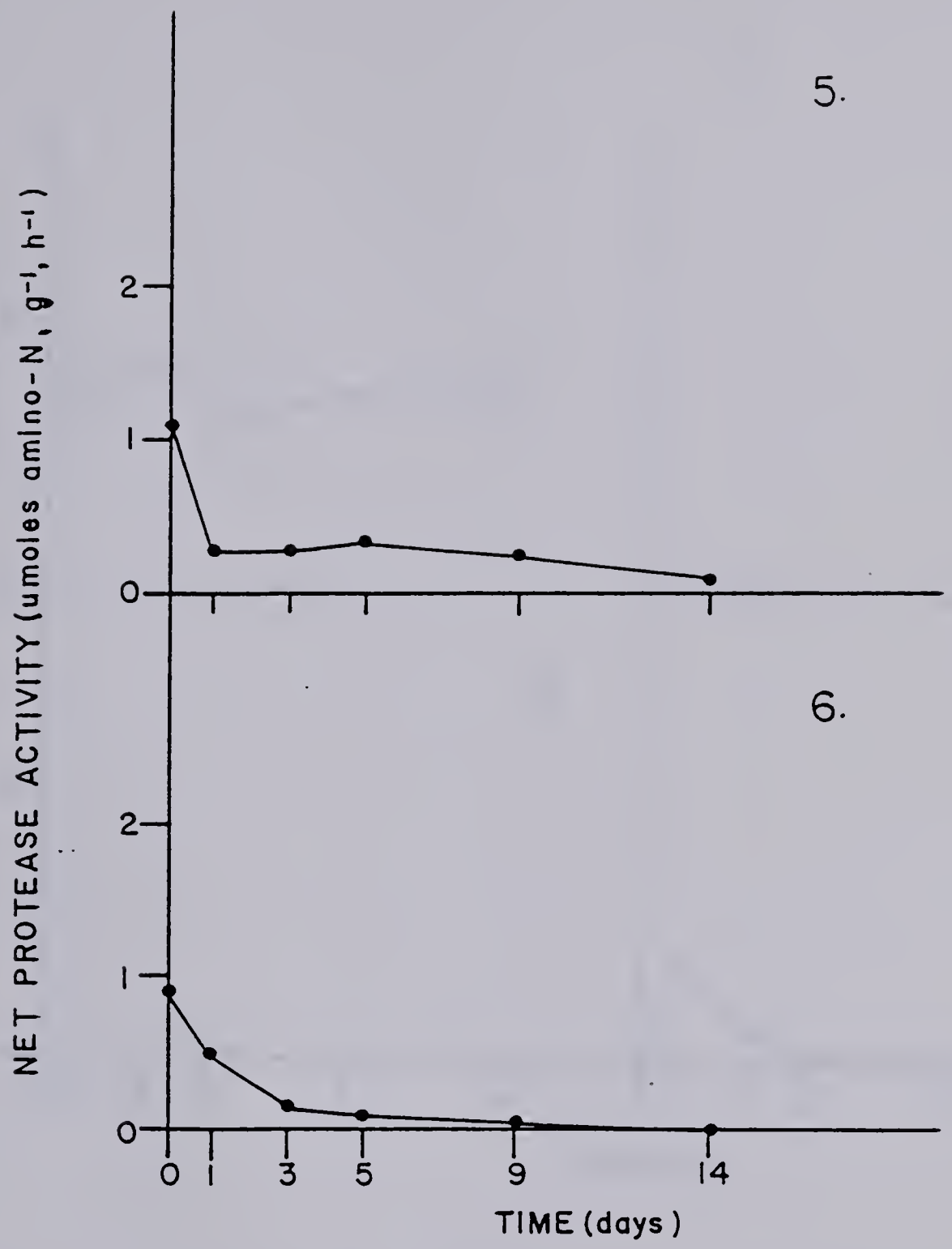

Figure 5.6 Net protease activity in Breton soil amended with;
5. glycyl-l-leucine and glucose
6. glycylglycine and glucose.

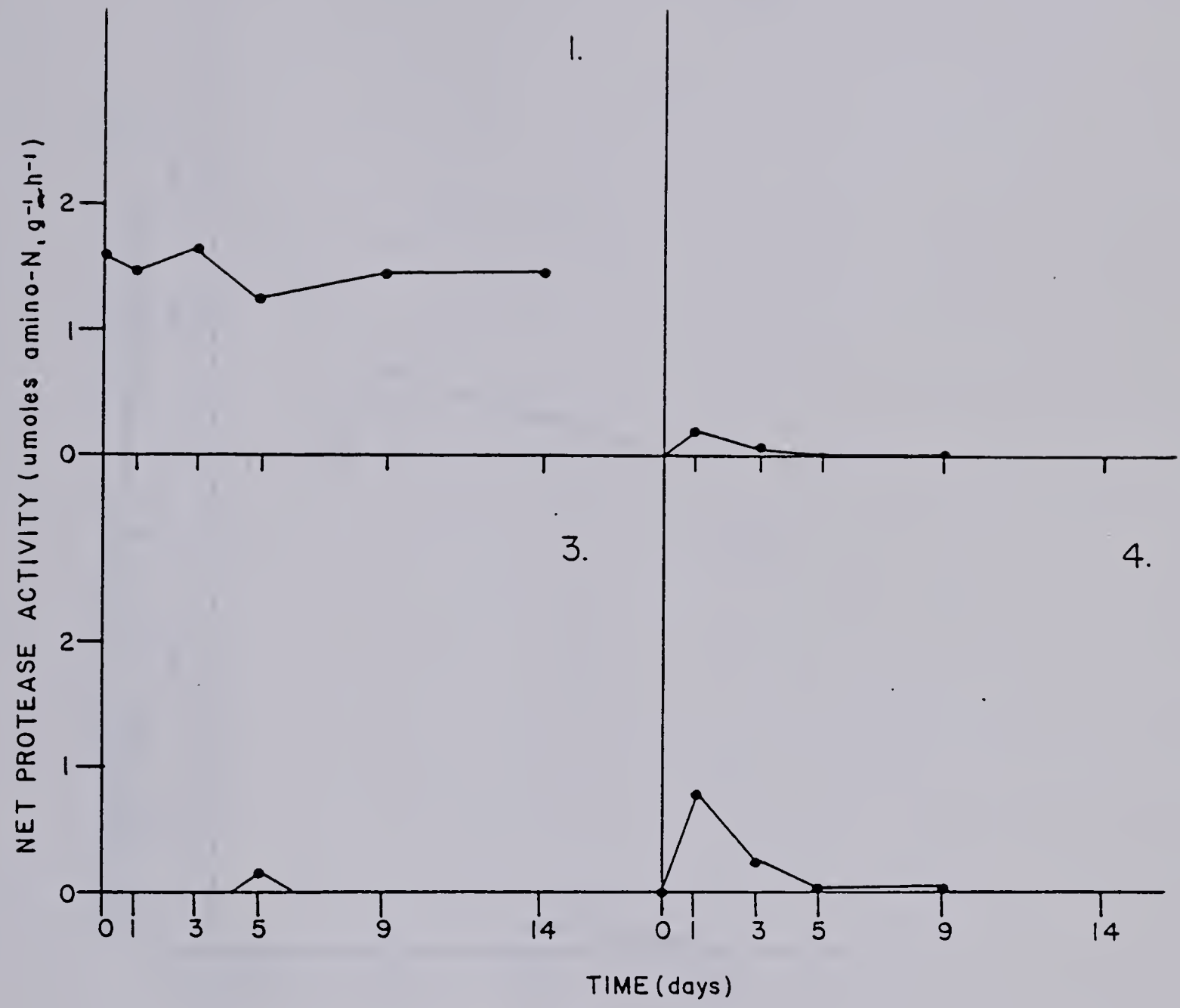

Figure 5.7 Net protease activity in Malmo soil unamended (control) and amended with amino acids:

1. control
2. leucine and glucose
3. lysine and glucose
4. phenylalanine and glucose.

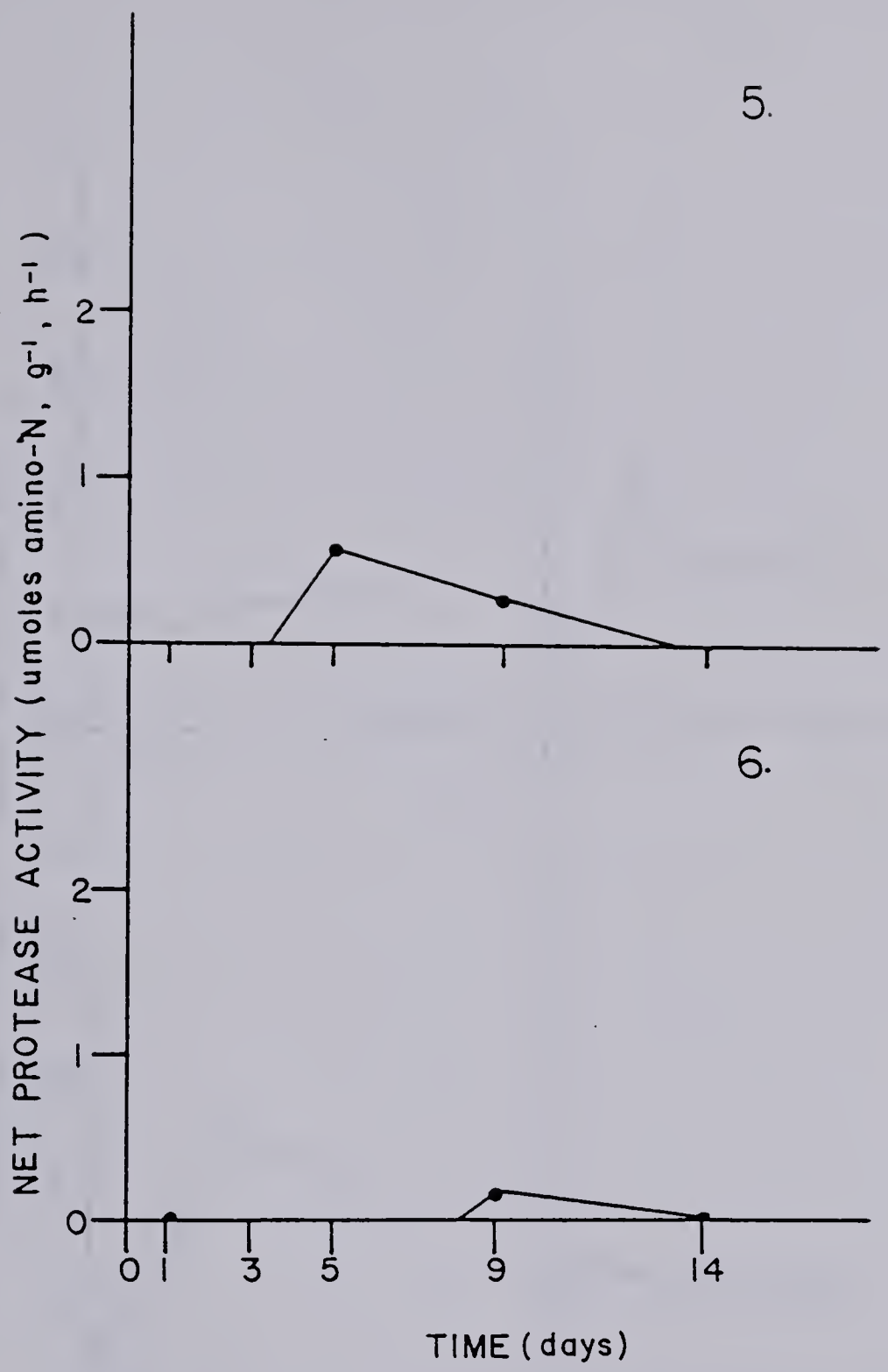

Figure 5.8 Net protease activity in Malmo soil amended with;
5. glycyl-l-leucine and glucose
6. glycylglycine and glucose.

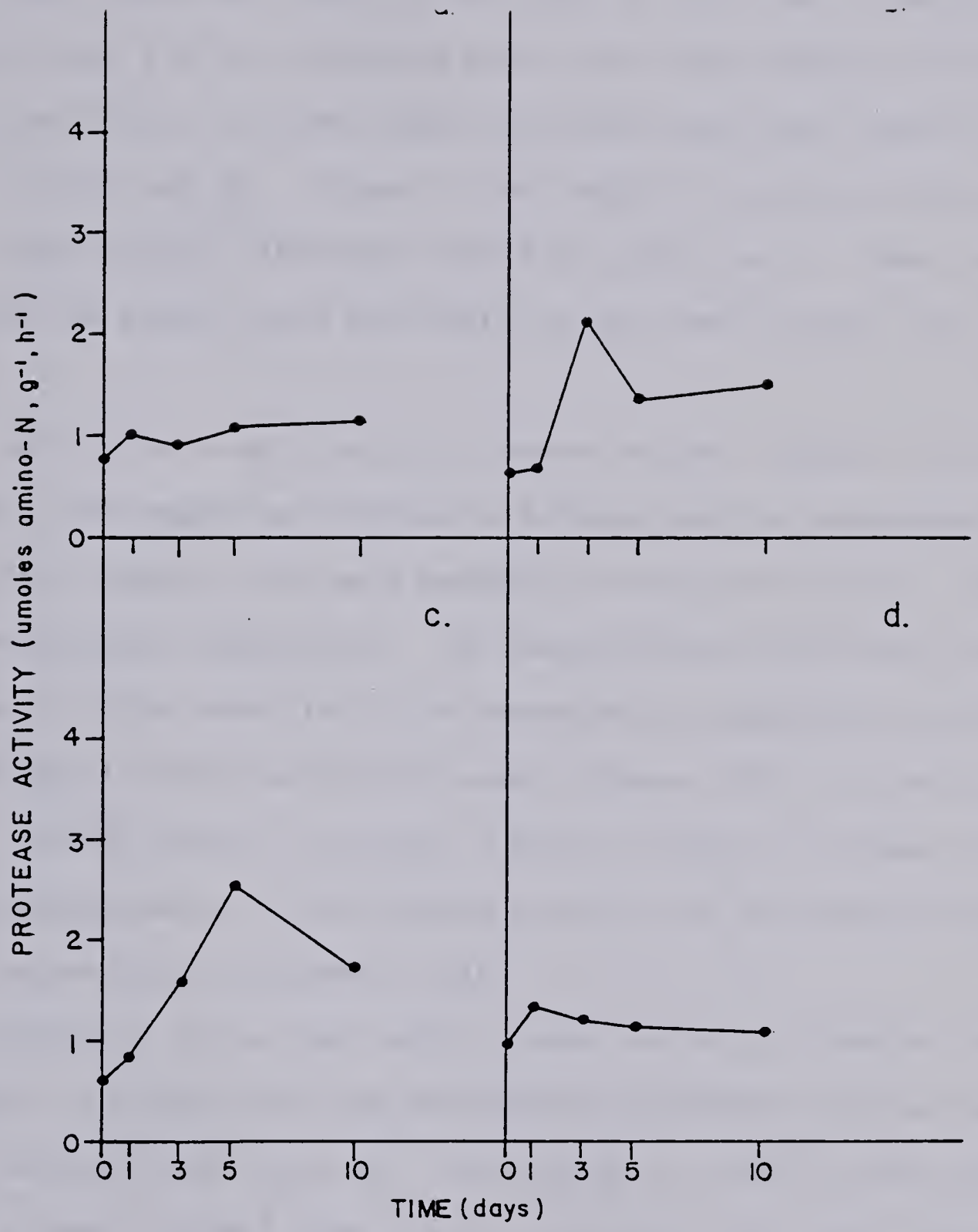

Figure 5.9 Protease activity in a Malmo soil.

(a) control

(b) casein control

(c) casein and rifampicin

(d) rifampicin control

measured protease activity by rifampicin alone in the first five days of the incubation period.

When casein was added to the soil on day 0 but rifampicin was added on day 3 of the incubation period the results (Figure 5.10a) were not significantly different from those with casein alone ($p=0.01$).

Glucose and NO_3^- (Figure 5.10d) failed to produce results that were significantly different from the control soil. These results confirm the results noted previously for the same treatment (Sec. 3 and 4).

Addition of c-AMP along with glucose and NO_3^- (Figure 5.10b) gave results that suggest an increase in protease activity approaching that found when casein is the soil amendment although this was not found to be statistically significant. The characteristic peak value occurring on day 3 of the incubation period corresponds in magnitude and time of appearance to those results for casein (Figure 5.9b). A c-AMP control soil produced results that showed a small increase in protease activity occurring on day 1 of the incubation period and declining to control soil values by day 3 (Figure 5.11d).

Amendment of the soil with glucose and NH_4^+, produced results (Figure 5.11a) that were not significantly different from the control soil moistened with H_2O only. The addition of c-AMP to soils amended with glucose and NH_4^+ gave results consistent with c-AMP alone; (1) there was a small increase in activity, and (2) the increase occurred during the first day (Figure 5.11b).

Dibutyryl c-AMP when incubated with soils amended either with glucose and NO_3^- or glucose and NH_4^+, gave results that were not significantly different from the control soil (Figures 5.11c, and 5.10c).

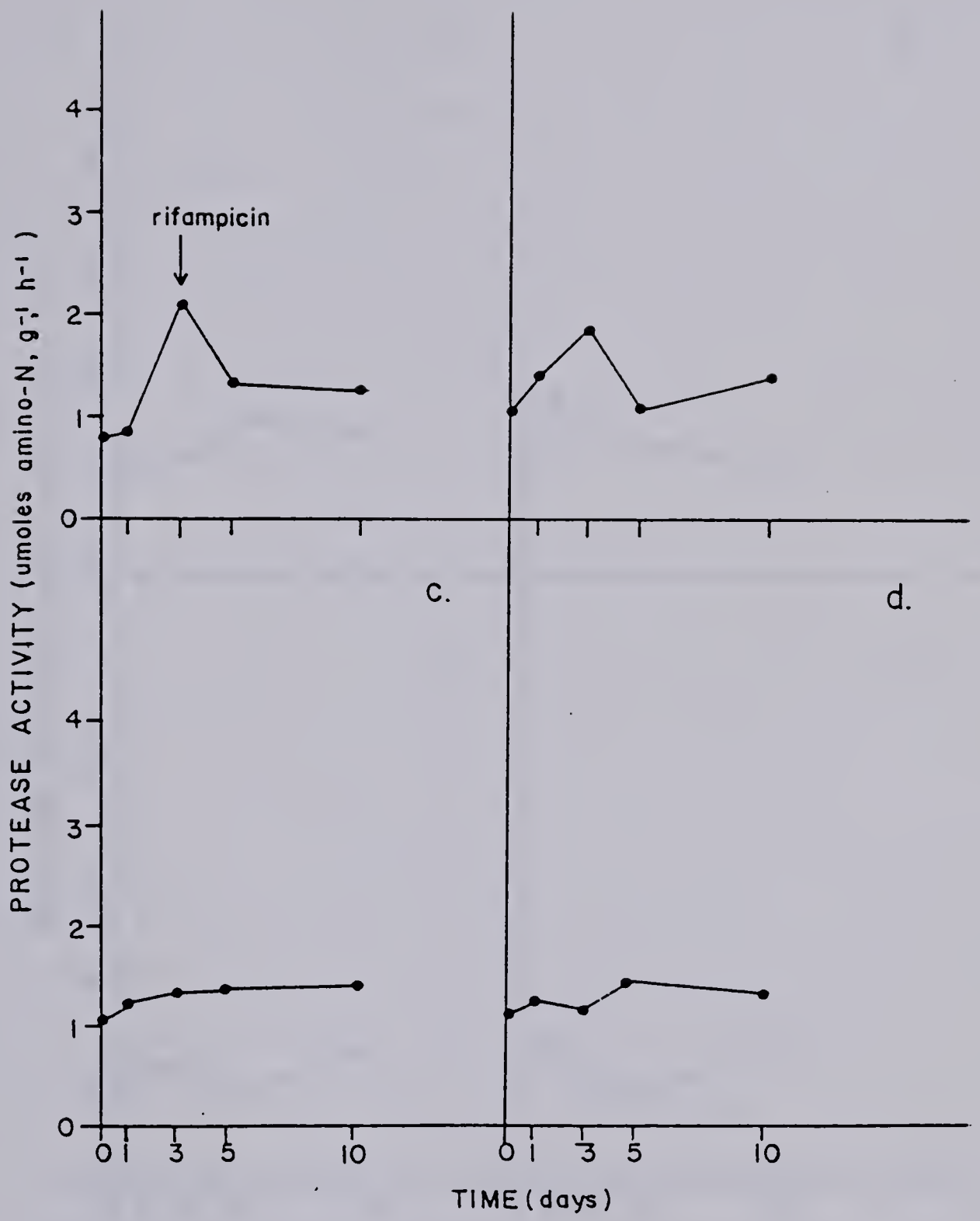

Figure 5.10 Protease activity in a Malmo soil amended with:
(a) casein and rifampicin (added on day 3.)
(b) glucose, KNO_3 and c-AMP
(c) glucose, KNO_3 and di-but-c-AMP.
(d) glucose and KNO_3.

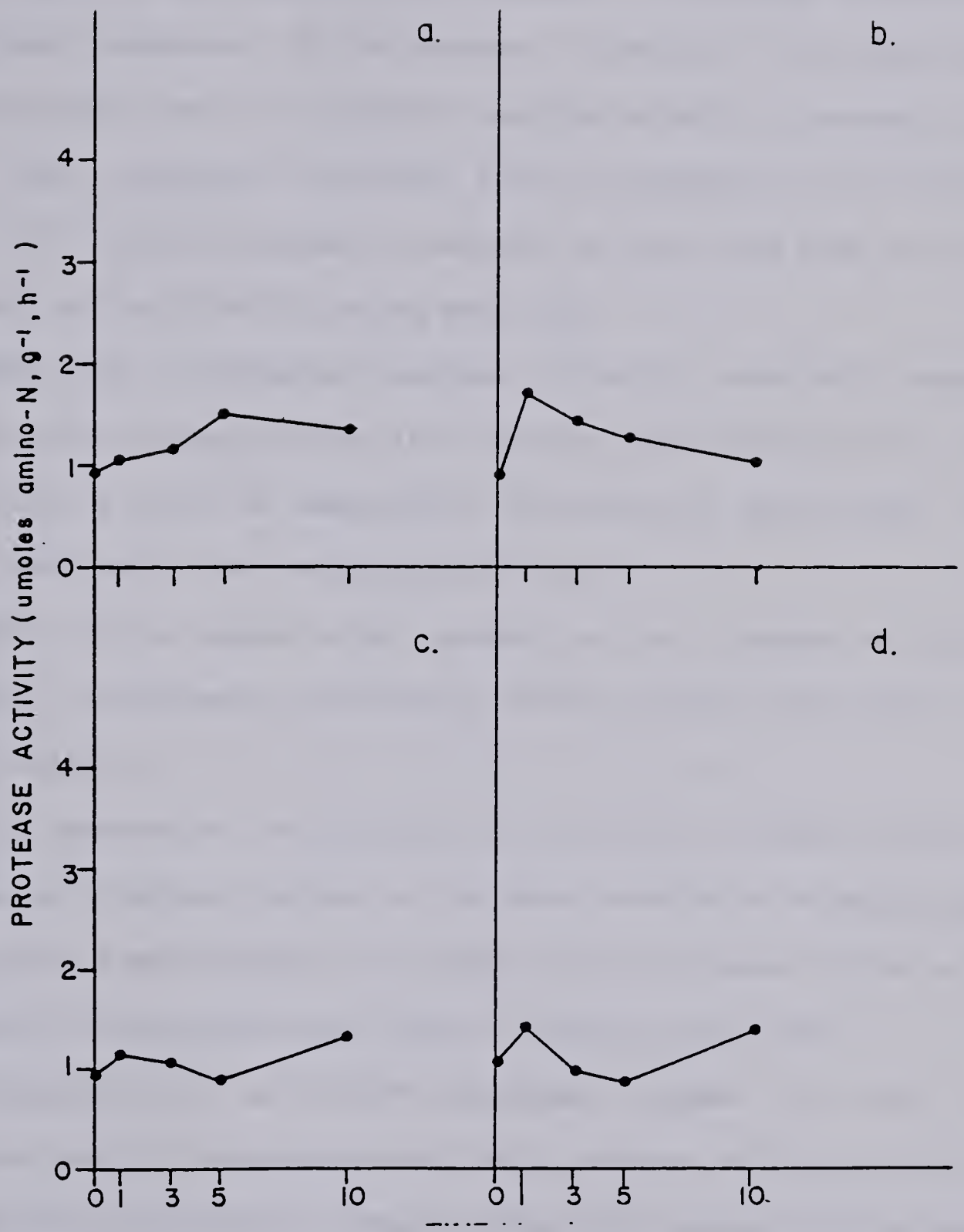

Figure 5.11 Protease activity in a Malmo soil, amended with:

(a) glucose and NH_4^+

(b) glucose and NH_4^+ and c-AMP

(c) glucose and NH_4^+ and di-but-c-AMP

(d) c-AMP control.

5.4 Discussion

In experiments using amino acids and dipeptides, the control soils, moistened only with H_2O were assayed for protease activity with and without substrate. In the absence of substrate there was a small but measureable level of ninhydrin reactive material, presumably amino acids, that remained relatively stable throughout the incubation period. An initial increase in activity on day 0 has been previously discussed as the effect of adding extra H_2O.

Amino-acid or dipeptide amendment of soils, especially amendment with leucine or phenylalanine, indicated the assay system used in these studies has a built in sensitivity to particular amino acids. Most of the added amino-N was metabolized by day 5.

None of the amino acids tested in the presence of glucose increased, or decreased, measured protease activity over that in the control soils.

This observation is supported by the work of Lasure (1980) who reported a stimulatory effect on protease production by Mucor miehei (zygomycete) when provided with casein but no protease synthesis when provided with individual amino acids or casein hydrolysate.

Nannipierri et al (1979) measured changes in amino acid concentrations in non-preincubated soils amended with a glucose and sodium nitrate solution and compared them with control soils moistened with H_2O only. The results of their experiments showed that total acid-soluble amino acids tended to increase over the period day 0 to day 3, declining thereafter to levels not significantly different from the control soil. Measurements of protease activity towards casein as an assay substrate, in a glucose and NO_3^- amended soil (Nannipierri et al 1979) showed that protease activity increases after the decline in

acid-soluble amino acid measurements. This increase in protease activity also coincides with the phase of rapid decline in numbers of viable bacteria. The authors state that: "During the death of the biomass a large number of proteins are available and, consequently it is during this phase that surviving microorganisms presumably produce large amounts of proteolytic enzymes." A cause and effect relationship is implicit in this statement.

The results of Nannipierri et al (1979) confirm those of Ladd and Paul (1973) who also found a short-lived increase in protease activity coinciding with the decline in viable microorganisms following exhaustion of a glucose amendment. The work of Nannipierri et al (1979) and Ladd and Paul (1973) support the hypothesis that production of proteolytic enzymes by soil microorganisms is initiated by the presence of proteins. This hypothesis is further confirmed here by the increase in measured protease activity upon incubation of casein with the soils used. The present results do not fully support the observations of Nannipierri et al (1979) and Ladd and Paul (1973), with respect to amendment of soils with glucose and NO_3^--N. Repeated experiments using glucose and NO_3^- amendment of the Breton and Malmo soils failed to produce results indicating any major change in measured protease activity over the incubation period of 14 days. Thus an increase in the microbial population followed by a subsequent rapid decline in numbers of bacteria, due to exhaustion of recently supplied substrate, does not necessarily result in an increase in protease activity.

Implicit in the statement of Nannipierri et al (1979) is the phenomenon of opportunism expressed by the biomass in responding to the

abundant supply of proteins resulting from the rapid decline in viable bacteria. This lends credence to the concept of "biological conservation" as well as supporting the hypothesis that the soil biomass responds to a signal(s) in the environment that triggers the production of extracellular proteolytic enzymes, since the response is a function of the appearance of proteins and not an increase in cell numbers.

Burns (1982) points out "... that soils, with their many different sites of enzyme activity, have a number of ways of responding to each substrate. For example, the initial catalysis of a substrate may be the function of the extracellular immobilized enzymes .... whilst microbial response may occur subsequently and only if substrate levels are sufficiently elevated. This suggests that soil microorganisms may have an integrated rather than a casual relationship with extracellular immobilized enzymes ...". The model of Burns (1982) accounts not only for the steady state level of exoenzyme activity but also the induction of new enzymes as a result of substrate hydrolysis, and the subsequent decline in activity resulting from a decrease in available substrate. The model allows an interpretation of the glucose and NO_3^--N amended soil data of Ladd and Paul (1973) and Nannipierri et al (1979) based on their observations that the increase in measured protease activity occurs after the decline in viable bacterial numbers. It does not, however, accommodate the results from the present study.

In experiments designed to examine the nature of intracellular regulation of protease synthesis, control soils gave results consistent with previous trials using the same soils (sections 3 & 4) moistened with water only. Addition of casein to the soils produced a short

lived two-fold increase in measured protease activity having a maximum on day 3 of the incubation period. These results further confirm previous findings (sections 3 & 4).

When rifampicin (a transcriptional inhibitor) was included with the casein treatment, a broadening of the peak of measured protease activity was observed with the peak maxima occurring on day 5 of the incubation period, rather than on day 3 as had been previously observed for casein alone. Statistical calculations reveal that the activity values on day 3 for the two treatments were significantly different ($p=0.05$). Whilst a single replicated test in not conclusive, the data suggest that protease synthesis has been delayed or at least partially inhibited for a period of two days following incubation of the soil with casein and rifampicin. The observation that the delay period is for two days suggests that the added rifampicin may be subject to microbial decomposition with subsequent removal of the inhibiting effect of the rifampicin. Decomposition of rifampicin would likely begin immediately after its addition to soil and progress over the two day lag period observed. This would account for the lower activity level on day 3 since the inhibiting effect would not be expected to be total if insufficient rifampicin remained at that time.

When rifampicin was added to the soil to coincide with the occurrence of the expected peak maxima no inhibition effect was observed. This suggests that once synthesis has been initiated it is short lived and the new enzymes formed, as a result of stimulatory signal(s) associated with casein amendment, are either mineralized, or inactivated, and that the stimulatory signals(s) is no longer present in the soil environment.

Bromke and Hammel (1979) working with S. marcescens in pure culture observed that gelatin-induced protease synthesis was inhibited by rifampicin at 10^{-4}M. Their hypothesis was that gelatin was an inducer for a specific group of proteases and that new protease specific mRNA would be synthesized in the presence of gelatin. Incubation of the organism with both gelatin and rifampicin resulted in minimal growth and minimal synthesis of protease. It is evident from their results that S. marcescens was incapable of producing significant amounts of gelatin specific protease in the presence of rifampicin. Incubation of S. marcescens on gelatin with glycerol added gave results that showed an increase in growth but a decrease in specific activity of protease. The glycerol effect was shown to be due to a repression of enzyme synthesis eliminated by addition of 5mM dibutyryl c-AMP. Thus two distinct regulatory systems could be demonstrated in S. marcescens with respect to exoprotease synthesis. The first, induction by gelatin, is linked directly to regulation at the level of transcription of the mRNA specific for the induced enzyme; and the second catabolite repression of enzyme synthesis which was observed by incubation of the cells with glycerol followed by derepression using exogenous c-AMP.

The duality of exoenzyme control suggested by the data of Bromke and Hammel (1979) indicates that hydrolysis of proteinaceous substances may be carried out by microorganisms to supply both a carbon and a nitrogen source and is not only the result of a search for carbon as an energy source. Thus it appears that, at least for some microorganisms, the presence in the environment of an inducer molecule will result in the production of exoenzymes specific for that inducer,

albeit in reduced quantity whether or not glucose is present as well. In the presence of glucose alone microorganisms are repressed for protease production and no exoproteases are synthesized. The data of Bromke and Hammel (1979) suggests a decrease, but not a complete absence, of protease synthesis when a readily metabolizeable carbon source is added to a protease producing population of cells. From their data it would appear that the extent of decrease in protease synthesis is related to the concentration of added carbon source.

Further evidence of a dual control regimen for exoenzyme synthesis is suggested by the report of Klapper et al (1973) and Shinmyo et al (1978) working with Aspergillus oryzae and Aspergillus niger respectively. In both reports, catabolite repression of exoprotease synthesis is related to the concentration of the newly added carbon source to a population of cells already induced for exoprotease synthesis.

These reports are consistent with the present observations regarding rifampicin and c-AMP effects in soil. When glucose and NO_3^- or glucose and NH_4^+ are added to the soil, little consistent difference from the control soils was observed. When c-AMP was added along with glucose and NO_3^- a short lived increase of approximately two-fold was observed in protease activity. Although not statistically significantly different from the control, this increase equalled that obtained using casein. The data are consistent with the hypothesis that a reversible catabolite repression control mechanism to regulate protease production is expressed in soil systems. Dibutyryl c-AMP did not produce results that were significantly different from the control soil, which may be an indication of the selectivity of transport

mechamism(s) in the cell membranes.

Addition of c-AMP, or dibutyryl c-AMP, along with glucose and NH_4^+ did not produce results that were significantly different ($p=0.05$) from the c-AMP control soil. No peak of activity was observed on day 3 for either treatment tested. This latter observation is evidence that in the glucose and NH_4^+ amended soils, regulation of protease synthesis is not due solely to catabolite repression. The results of the glucose and NO_3^- and c-AMP amended soils however suggest that catabolite repression may be at least partially expressed in the regulation of protease synthesis in the soils used. It would appear that NO_3^- and NH_4^+ behave differently in the soils examined, when c-AMP is used to derepress enzyme synthesis. The difference between NO_3^- and NH_4^+ when incubated with glucose and c-AMP requires further study.

5.5 Summary

The addition of the amino acids leucine, lysine, phenylalanine or either of the two dipeptides glycyl-L-leucine or glycylglycine, along with glucose, to preincubated Malmo and Breton soils at the concentrations used did not produce results that were indicative of stimulation of protease synthesis. Blanks gave results that showed a difference in the senstivity of the ninhydrin reagent to the amino acids used. The results of the blanks also showed that initially high levels of measured activity were due to the addition of the amino acids and not to protease activity per se.

The results of Nannipierri et al (1979), Ladd and Paul (1973) and those of this study suggest that, at least one form of protease synthesis regulation in soil is linked to the presence of proteins in

the cellular environment. Experiments carried out in this study using rifampicin suggest a control at the level of transcription. Protease synthesis was shown to be partially inhibited when casein and rifampicin were added to the soil. The observed inhibition was short-lived and statistically significant. The data suggest that rifampicin is decomposed quite rapidly in the soils used. Adding rifampicin on day 0 along with casein results in only partial inhibition at the level used. Addition of rifampicin on day 3 produces no observable inhibition of the expected increase in protease activity. This confirms the hypothesis that the increase in protease activity observed upon amendment of the soil with casein is short lived and is in fact a response to the added protein. Once the initiation of new protease synthesis is complete, the transcription inhibitor rifampicin is no longer effective, presumably because no significant induction of the enzyme is taking place.

The possibility of catabolite repression of exoprotease synthesis also being a contributing regulatory mechanism is supported by the finding that c-AMP appears to derepress protease synthesis in soils amended with glucose and NO_3^-. Soils amended with glucose and NH_4^+, however did not produce results that would indicate derepression of protease synthesis by c-AMP. For both treatments, (Glucose and NO_3^--N or glucose and NH_4^+) dibutyryl-cAMP was not effective in derepressing protease synthesis.

In a heterogeneous microbial population such as exists in soils conditions in microenvironmental loci will impinge on the cells within those loci. The data from this study have shown that there may be at least two regulatory mechanisms controlling protease synthesis in soil.

The method of measurement used yields only gross protease activity, and as such it is the cumulative activity of cells of differing type under a range of microenvironmental conditions. Bromke and Hammel (1979) have shown in pure culture that within a single species there can be two regulatory mechanisms controlling the synthesis of exoprotease enzymes. The interplay of environmental conditions and multiple regulatory mechanisms makes it difficult to demonstrate the existence of any one system in isolation. The evidence gathered in this study has demonstrated a clear trend indicating that catabolite repression and induction are two possible means of control of exoprotease synthesis in the soils used.

The existence of at least two regulatory mechanisms for control of protease synthesis in soil microorganisms, is evidence to support the hypothesis that conservation of energy and materials is expressed in soil systems is a manner similar to, if not the same, as in pure cultures.

6. General Summary and Conclusions

6. General Summary and Conclusions

Short term assays using either casein or the dipeptide derivative CBZ-PL were used to quantify changes in protease activity of the Malmo and Breton soils in relation to amendment separately with casein, glucose, glucose and ammonia, glucose and NO_3^- or NH_4^+. Amendment of the soils with NH_4^+ required the use of an NH_4^+ removal step in the assay procedure to prevent its reaction with ninhydrin in the final colourimetric determination of the amino-acid end product. The NH_4^+ removal technique was used throughout and gave good results when all controls, blanks, treatment and standards were treated.

With the exception of casein, none of the treatments produced major increases in protease activity relative to controls with either assay substrate used. The use of NH_4^+ at concentrations up to 1000 mg kg^{-1} in the assay or incubated with the soil, failed to produce results that would indicate any effect of NH_4^+ on protease activity or synthesis.

Amendment of both soils with casein produced a short-lived increase in protease activity of at least two fold. The increase in activity was not substantially influenced by addition of glucose, plus NH_4^+ or NO_3^- and casein. The Malmo soil had a greater activity than the Breton soil. The two soils differed in apparent substrate preference; with the Malmo soil more active against CBZ-PL and the Breton soil more active against casein. There was a greater difference between the activity values obtained using the two substrates in the Malmo than in the Breton soil. The differences in overall activity values may be due to differences in: indigenous biomass levels in the two soils; activity peak appearance between the two substrates;

microbial species substrate specificity.

Experiments using preincubated and non-preincubated soils, employing glucose and glucose and NO_3^- as soil amendments, failed to yield increases in activity over the control soils. The lack of any major effect of adding non-proteinaceous compounds to soil was attributed to the existence of intracellular controls that regulated protease synthesis in the absence of a specific requirement for those enzymes, possibly a form of catabolite repression.

Amendment of both soils with glucose plus a limited number of individual amino-acids and dipeptides separately had no significant effect on protease synthesis, indicating that at the level used these potential decomposition products of proteolysis are not effective stimulatory signals in exoprotease synthesis.

Experiments using rifampicin suggest that exoprotease synthesis in soils may be controlled at the level of transcription. Other experiments using c-AMP provided evidence suggesting the possible role of catabolite repression in controlling exoprotease synthesis in soils. Collectively, the data are consistent with the hypothesis that at least two regulatory mechanisms governing extracellular protease synthesis are expressed in soil systems.

There exists the possiblity that these measurements reflect protease production of several genera of microorganisms, some of which are inducible for de novo protease synthesis, and some of which are constitutive for protease production but are subject to catabolite repression or possibly end-product inhibition. In the absence of genus specific data, or the ability to discriminate between two or more pools of newly synthesized enzymes this question was not resolved.

at this time.

This study has shown that it is possible to examine the nature of intracellular control of protein synthesis, specifically exoprotease synthesis, in a heterogeneous microbial population in soil under laboratory conditions. Although only gross measurements of activity are possible at this time, extrapolations from pure culture studies are of value in interpretation of soil enzyme studies. Catabolite repression as a control mechanism and the demonstration of partial inhibition of protease synthesis with a transcription inhibitor, in an environment shown previously to be conducive to protease synthesis, suggests that microorganisms when in soil are subject to the same kinds of controls evidenced by pure cultures in vitro. The dynamics of exchange and transport of molecules within soils are predisposing factors that presently limit the elucidation of specific requirements, (ie: ion concentrations, pH optima, inducer(s) and transport of inducer(s) across the cell wall) and conditions necessary to achieve a complete picture of a well coordinated system of controls within soil at the cellular level.

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APPENDICES

APPENDIX A

CHARACTERISTICS OF CULTIVATED SITES

TABLE 1. Description of sites and soils studied.

Site Characteristic	Site: Ellerslie	Site: Breton
Legal location	NE-24-51-25-W4th	NE-25-47-4-W5th
Soil zone	Black	Gray Luvisol
Soil association	Malmo	Breton
Dominant sub-group profile	Orthic Black Chernozemic	Orthic Gray Luvisolic
landform	gently undulating	rolling morainal
slope class	2%	2-5%
native vegetation	Populus balsamifera Cornus stolonifera	Populus tremuloides Picea glauca
elevation above sea level	686 m.	850 m.
pH	6.3	6.0
%C	5.3a	1.36
%N	0.54a	0.12
Texture	Silty clay loam	Silt loam

a: Monreal, M. 1983. Denitrification and its relation to soluble carbon. MSc Thesis University of Alberta, Edmonton, Canada

APPENDIX B

TABLE 1. NaOH addition prior to heating to drive off ammonia

Sample size(ml)	H_2O(ml)	5N NaOH(ml)
0.1	0.9	0.2
0.5	0.5	0.2
1.0	0.0	0.4

The amount of 5N NaOH required to raise the pH to $\geq$ 10 is dependent on the volume of supernatant sample chosen for the assay. The greater the sample size, the larger will be the volume of the buffer contained therein. If dilutions are made of the sample, a non-linear dilution relationship was found with respect to the amount of 5N NaOH required.

VARIABLE VAR4 CBZ
BY VARIABLE VAR3 TRT

ANALYSIS OF VARIANCE

SOURCE	D.F.	SUM OF SQUARES	MEAN SQUARES	F RATIO	F PROB.
BETWEEN GROUPS	5	121.0704	24.2141	6.688	0.0000
WITHIN GROUPS	83	300.4971	3.6204		
TOTAL	88	421.5674			

GROUP	COUNT	MEAN	STANDARD DEVIATION	STANDARD ERROR	MINIMUM	MAXIMUM	95 PCT CONF INT FOR MEAN
GRP1	14	1.1550b	0.7442	0.1989	0.2500	2.2200	0.7253 TO 1.5847
GRP2	15	1.1967b	0.6426	0.1659	0.4500	2.3400	0.8408 TO 1.5525
GRP3	15	1.3320b	0.5439	0.1404	0.4800	2.5800	1.0308 TO 1.6332
GRP4	15	3.6567a	3.1360	0.8097	0.4100	9.8900	1.9200 TO 5.3933
GRP5	15	3.7327a	3.1728	0.8192	0.4300	9.8900	1.9756 TO 5.4897
GRP6	15	1.2327b	0.5829	0.1505	0.4000	2.2400	0.9098 TO 1.5555
TOTAL	89	2.0610	2.1887	0.2320	0.2500	9.8900	1.5999 TO 2.5221
	FIXED EFFECTS MODEL		1.9027	0.2017			1.6599 TO 2.4622
	RANDOM EFFECTS MODEL	0.5218					0.7198 TO 3.4022

RANDOM EFFECTS MODEL - ESTIMATE OF BETWEEN COMPONENT VARIANCE 1.3885

TESTS FOR HOMOGENEITY OF VARIANCES

COCHRANS C = MAX. VARIANCE/SUM(VARIANCES) = 0.4681, P = 0.000 (APPROX.)
BARTLETT-BOX F = 18.602. P = 0.000
MAXIMUM VARIANCE / MINIMUM VARIANCE = 34.033

SOIL: MALMO
SECTION 3.3: ASSAY SUBSTRATE CBZ-PL; a,b = values of protease activity significantly different at P = 0.05

VARIABLE VAR5 CAS
BY VARIABLE VAR3 TRT

ANALYSIS OF VARIANCE

SOURCE	D.F.	SUM OF SQUARES	MEAN SQUARES	F RATIO	F PROB.
BETWEEN GROUPS	5	21.2389	4.2478	9.181	0.0000
WITHIN GROUPS	83	38.4003	0.4627		
TOTAL	88	59.6392			

GROUP	COUNT	MEAN	STANDARD DEVIATION	STANDARD ERROR	MINIMUM	MAXIMUM	95 PCT CONF INT FOR MEAN
GRP1	14	0.5071 b	0.1563	0.0418	0.2100	0.7100	0.4169 TO 0.5974
GRP2	15	0.7240 b	0.3157	0.0815	0.2200	1.2500	0.5491 TO 0.8989
GRP3	15	0.7953 b	0.3256	0.0841	0.2200	1.3600	0.6150 TO 0.9756
GRP4	15	1.7040 a	1.0833	0.2797	0.2100	3.4000	1.1041 TO 2.3039
GRP5	15	1.7580 a	1.1173	0.2885	0.2100	3.8400	1.1392 TO 2.3768
GRP6	15	0.8460 b	0.3042	0.0785	0.2200	1.2300	0.6776 TO 1.0144
TOTAL	89	1.0619	0.8232	0.0873	0.2100	3.8400	0.8885 TO 1.2353
FIXED EFFECTS MODEL			0.6802	0.0721			0.9185 TO 1.2053
RANDOM EFFECTS MODEL			0.2185				0.5001 TO 1.6237

RANDOM EFFECTS MODEL - ESTIMATE OF BETWEEN COMPONENT VARIANCE 0.2552

TESTS FOR HOMOGENEITY OF VARIANCES

COCHRANS C = MAX. VARIANCE/SUM(VARIANCES) = 0.4549, P = 0.000 (APPROX.)
BARTLETT-BOX F = 14.891, P = 0.000
MAXIMUM VARIANCE / MINIMUM VARIANCE = 51.088

SOIL: MALMO
SECTION 3.3 ASSAY SUBSTRATE CASEIN

VARIABLE VAR4 CBZPL
BY VARIABLE VAR3 SAMPLING TIME

ANALYSIS OF VARIANCE

SOURCE	D.F.	SUM OF SQUARES	MEAN SQUARES	F RATIO	F PROB.
BETWEEN GROUPS	5	26.6486	5.3297	5.453	0.0002
WITHIN GROUPS	100	97.7359	0.9774		
TOTAL	105	124.3845			

GROUP	COUNT	MEAN	STANDARD DEVIATION	STANDARD ERROR	MINIMUM	MAXIMUM	95 PCT CONF INT FOR MEAN
GRP1	17	2.4865a	0.5928	0.1438	1.4800	3.3300	2.1817 TO 2.7912
GRP2	18	3.7828b	1.2014	0.2832	2.2300	7.2600	3.1853 TO 4.3802
GRP3	18	3.0950b	0.6131	0.1445	2.1100	4.1100	2.7901 TO 3.3999
GRP4	17	3.8771b	1.2717	0.3084	2.1100	6.2500	3.2232 TO 4.5309
GRP5	18	3.7161a	1.0165	0.2396	2.3400	6.0100	3.2106 TO 4.2216
GRP6	18	3.8039b	1.0217	0.2408	2.3200	5.5700	3.2958 TO 4.3120
TOTAL	106	3.4655	1.0884	0.1057	1.4800	7.2600	3.2559 TO 3.6751
	FIXED EFFECTS MODEL		0.9886	0.0960			3.2750 TO 3.6560
	RANDOM EFFECTS MODEL		0.2243				2.8889 TO 4.0421

RANDOM EFFECTS MODEL - ESTIMATE OF BETWEEN COMPONENT VARIANCE 0.2464

TESTS FOR HOMOGENEITY OF VARIANCES

COCHRANS C = MAX. VARIANCE/SUM(VARIANCES) = 0.2757, P = 0.170 (APPROX.)
BARTLETT-BOX F = 3.102, P = 0.009
MAXIMUM VARIANCE / MINIMUM VARIANCE = 4.602

SECTION 4.3: SERIES I (MALMO)
ASSAY SUBSTRATE = CBZ-PL

VARIABLE VAR4 CBZPL
BY VARIABLE VAR3 SAMPLING TIME

ANALYSIS OF VARIANCE

SOURCE	D.F.	SUM OF SQUARES	MEAN SQUARES	F RATIO	F PROB.
BETWEEN GROUPS	5	34.1423	6.8285	6.232	0.0000
WITHIN GROUPS	102	111.7681	1.0958		
TOTAL	107	145.9104			

GROUP	COUNT	MEAN	STANDARD DEVIATION	STANDARD ERROR	MINIMUM	MAXIMUM	95 PCT CONF INT FOR MEAN
GRP1	18	0.9578 a	0.3632	0.0856	0.4400	1.6900	0.7772 TO 1.1384
GRP2	18	2.0911 b	1.1563	0.2725	0.6700	4.4700	1.5161 TO 2.6661
GRP3	18	1.0900 b	0.3224	0.0760	0.6200	1.7900	0.9297 TO 1.2503
GRP4	18	1.5428 b	0.6483	0.1528	0.7700	2.6400	1.2204 TO 1.8651
GRP5	18	1.9994 a	1.5578	0.3672	0.5800	5.0300	1.2248 TO 2.7741
GRP6	18	2.5406 b	1.4679	0.3460	0.7800	4.6700	1.8106 TO 3.2705
TOTAL	108	1.7036	1.1678	0.1124	0.4400	5.0300	1.4809 TO 1.9264
	FIXED EFFECTS MODEL		1.0468	0.1007			1.5038 TO 1.9034
	RANDOM EFFECTS MODEL			0.2514			1.0572 TO 2.3500

RANDOM EFFECTS MODEL - ESTIMATE OF BETWEEN COMPONENT VARIANCE 0.3185

TESTS FOR HOMOGENEITY OF VARIANCES

COCHRANS C = MAX. VARIANCE/SUM(VARIANCES) = 0.3691, P = 0.004 (APPROX.)
BARTLETT-BOX F = 12.554, P = 0.000
MAXIMUM VARIANCE / MINIMUM VARIANCE = 23.343

SECTION 4.3: SERIES I (BRETON)
ASSAY SUBSTRATE = CBZ-PL

VARIABLE VAR5 CASEIN
BY VARIABLE VAR3 SAMPLING TIME

ANALYSIS OF VARIANCE

SOURCE	O.F.	SUM OF SQUARES	MEAN SQUARES	F RATIO	F PROB.
BETWEEN GROUPS	5	25.2951	5.0590	8.588	0.0000
WITHIN GROUPS	97	57.1390	0.5891		
TOTAL	102	82.4341			

GROUP	COUNT	MEAN	STANDARD DEVIATION	STANDARD ERROR	MINIMUM	MAXIMUM	95 PCT CONF INT FOR MEAN
GRP1	18	0.3750 a	0.2343	0.0552	0.0500	0.7800	0.2585 TO 0.4915
GRP2	17	1.2182 b	0.8166	0.1981	0.0900	2.6700	0.7984 TO 1.6381
GRP3	18	0.7772 b	0.4246	0.1001	0.1000	1.5200	0.5661 TO 0.9884
GRP4	17	1.9612 b	1.2608	0.3058	0.1300	4.3300	1.3129 TO 2.6094
GRP5	17	1.3488 a	0.8212	0.1992	0.1300	2.5700	0.9266 TO 1.7710
GRP6	16	1.2125 b	0.6455	0.1614	0.1500	2.0900	0.8685 TO 1.5565
TOTAL	103	1.1371	0.8990	0.0886	0.0500	4.3300	0.9614 TO 1.3128

FIXED EFFECTS MODEL	0.7675	0.0756	0.9870 TO 1.2872
RANDOM EFFECTS MODEL		0.2218	0.5669 TO 1.7073

RANDOM EFFECTS MODEL - ESTIMATE OF BETWEEN COMPONENT VARIANCE 0.2605

TESTS FOR HOMOGENEITY OF VARIANCES

COCHRANS C = MAX. VARIANCE/SUM(VARIANCES) = 0.4437, P = 0.000 (APPROX.)
BARTLETT-BOX F = 8.942, P = 0.000
MAXIMUM VARIANCE / MINIMUM VARIANCE = 28.955

SECTION 4.3: SERIES I (MALMO)
ASSAY SUBSTRATE = CASEIN

VARIABLE VAR5 CASEIN
BY VARIABLE VAR3 SAMPLING TIME

ANALYSIS OF VARIANCE

SOURCE	D.F.	SUM OF SQUARES	MEAN SQUARES	F RATIO	F PROB.
BETWEEN GROUPS	5	26.1268	5.2254	6.087	0.0001
WITHIN GROUPS	101	86.7081	0.8585		
TOTAL	106	112.8349			

GROUP	COUNT	MEAN	STANDARD DEVIATION	STANDARD ERROR	MINIMUM	MAXIMUM	95 PCT CONF INT FOR MEAN
GRP1	18	0.6906 a	0.3302	0.0778	0.1600	1.2500	0.5264 TO 0.8547
GRP2	17	2.0059 b	1.2266	0.2975	0.3000	4.2500	1.3752 TO 2.6365
GRP3	18	0.9289 b	0.3367	0.0794	0.3000	1.4700	0.7614 TO 1.0963
GRP4	18	1.8228 b	1.1058	0.2606	0.1800	3.3900	1.2729 TO 2.3727
GRP5	18	1.7539 a	0.9398	0.2215	0.1400	3.0000	1.2865 TO 2.2212
GRP6	18	1.7467 b	1.1645	0.2745	0.2400	3.7800	1.1676 TO 2.3258
TOTAL	107	1.4866	1.0317	0.0997	0.1400	4.2500	1.2889 TO 1.6844
	FIXED EFFECTS MODEL		0.9266	0.0896			1.3089 TO 1.6643
	RANDOM EFFECTS MODEL		0.2210				0.9185 TO 2.0548

RANDOM EFFECTS MODEL - ESTIMATE OF BETWEEN COMPONENT VARIANCE 0.2449

TESTS FOR HOMOGENEITY OF VARIANCES

COCHRANS C = MAX. VARIANCE/SUM(VARIANCES) = 0.2899, P = 0.102 (APPROX.)
BARTLETT-BOX F = 9.070, P = 0.000
MAXIMUM VARIANCE / MINIMUM VARIANCE = 13.802

SECTION 4.3: SERIES I (BRETON)
ASSAY SUBSTRATE = CASEIN

VARIABLE VAR4 CBZPL
BY VARIABLE VAR3 TRT

ANALYSIS OF VARIANCE

SOURCE	D.F.	SUM OF SQUARES	MEAN SQUARES	F RATIO	F PROB.
BETWEEN GROUPS	3	3.1973	1.0658	19.390	0.0000
WITHIN GROUPS	55	3.0231	0.0550		
TOTAL	58	6.2204			

GROUP	COUNT	MEAN	STANDARD DEVIATION	STANDARD ERROR	MINIMUM	MAXIMUM	95 PCT CONF INT FOR MEAN
GRP1	15	2.4233 a	0.2867	0.0740	2.1900	3.2900	2.2645 TO 2.5821
GRP2	15	2.2800 a	0.2464	0.0636	1.9200	2.6500	2.1436 TO 2.4164
GRP3	15	2.7547 a	0.2116	0.0546	2.4000	3.0300	2.6375 TO 2.8718
GRP4	14	2.1214 a	0.1744	0.0466	1.8700	2.4800	2.0207 TO 2.2221
TOTAL	59	2.3995	0.3275	0.0426	1.8700	3.2900	2.3141 TO 2.4848
FIXED EFFECTS MODEL			0.2344	0.0305			2.3383 TO 2.4607
RANDOM EFFECTS MODEL				0.1345			1.9715 TO 2.8274

RANDOM EFFECTS MODEL - ESTIMATE OF BETWEEN COMPONENT VARIANCE 0.0685

TESTS FOR HOMOGENEITY OF VARIANCES

COCHRANS C = MAX. VARIANCE/SUM(VARIANCES) = 0.3770, P = 0.274 (APPROX.)
BARTLETT-BOX F = 1.145, P = 0.330
MAXIMUM VARIANCE / MINIMUM VARIANCE = 2.704

SECTION 4.3: SERIES II (MALMO)

VARIABLE VAR4 CBZPL
BY VARIABLE VAR3 TRT

ANALYSIS OF VARIANCE

SOURCE	D.F.	SUM OF SQUARES	MEAN SQUARES	F RATIO	F PROB.
BETWEEN GROUPS	3	3.7235	1.2412	29.318	0.0000
WITHIN GROUPS	53	2.2437	0.0423		
TOTAL	56	5.9672			

GROUP	COUNT	MEAN	STANDARD DEVIATION	STANDARD ERROR	MINIMUM	MAXIMUM	95 PCT CONF INT FOR MEAN
GRP1	14	1.6464 a	0.1299	0.0347	1.5100	1.9100	1.5714 TO 1.7214
GRP2	14	1.5007 a	0.2494	0.0667	1.2100	1.9100	1.3567 TO 1.6447
GRP3	14	1.9914 a	0.2688	0.0718	1.4900	2.4400	1.8362 TO 2.1466
GRP4	15	1.2933 a	0.1405	0.0363	1.1000	1.5900	1.2155 TO 1.3711
TOTAL	57	1.6025	0.3264	0.0432	1.1000	2.4400	1.5158 TO 1.6891
FIXED EFFECTS MODEL			0.2058	0.0273			1.5478 TO 1.6571
RANDOM EFFECTS MODEL				0.1477			1.1326 TO 2.0723

RANDOM EFFECTS MODEL - ESTIMATE OF BETWEEN COMPONENT VARIANCE 0.0842

TESTS FOR HOMOGENEITY OF VARIANCES

COCHRANS C = MAX. VARIANCE/SUM(VARIANCES) = 0.4224, P = 0.117 (APPROX.)
BARTLETT-BOX F = 3.459, P = 0.016
MAXIMUM VARIANCE / MINIMUM VARIANCE = 4.283

SECTION 4.3: SERIES II (BRETON)

VARIABLE VAR4 CBZPL
BY VARIABLE VAR3 TRT

ANALYSIS OF VARIANCE

SOURCE	D.F.	SUM OF SQUARES	MEAN SQUARES	F RATIO	F PROB.
BETWEEN GROUPS	5	17.5447	3.5089	4.542	0.0009
WITHIN GROUPS	102	78.7966	0.7725		
TOTAL	107	96.3413			

GROUP	COUNT	MEAN	STANDARD DEVIATION	STANDARD ERROR	MINIMUM	MAXIMUM	95 PCT CONF INT FOR MEAN
GRP1	17	2.4882 a	0.3607	0.0875	2.0300	3.1900	2.3028 TO 2.6737
GRP2	18	3.6317 a	1.4623	0.3447	2.2700	6.8500	2.9045 TO 4.3589
GRP3	18	2.8183 a	0.2652	0.0625	2.2700	3.2800	2.6864 TO 2.9502
GRP4	19	3.5663 a	1.1356	0.2605	2.4000	6.1600	3.0190 TO 4.1136
GRP5	18	2.9305 a	0.6133	0.1446	2.1100	4.4300	2.6255 TO 3.2356
GRP6	18	3.0644 a	0.7499	0.1767	2.3000	4.5200	2.6915 TO 3.4373
TOTAL	108	3.0932	0.9489	0.0913	2.0300	6.8500	2.9122 TO 3.2742
	FIXED EFFECTS MODEL		0.8789	0.0846			2.9255 TO 3.2610
	RANDOM EFFECTS MODEL		0.1803				2.6297 TO 3.5568

RANDOM EFFECTS MODEL - ESTIMATE OF BETWEEN COMPONENT VARIANCE 0.1521

TESTS FOR HOMOGENEITY OF VARIANCES

COCHRANS C = MAX. VARIANCE/SUM(VARIANCES) = 0.4682, P = 0.000 (APPROX.)
BARTLETT-BOX F = 11.845, P = 0.000
MAXIMUM VARIANCE / MINIMUM VARIANCE = 30.395

SECTION 5.3: SERIES I (MALMO)

VARIABLE VAR4 CBZPL
BY VARIABLE VAR3 TRT

ANALYSIS OF VARIANCE

SOURCE	D.F.	SUM OF SQUARES	MEAN SQUARES	F RATIO	F PROB.
BETWEEN GROUPS	5	23.7441	4.7488	3.118	0.0116
WITHIN GROUPS	102	155.3614	1.5232		
TOTAL	107	179.1055			

GROUP	COUNT	MEAN	STANDARD DEVIATION	STANDARD ERROR	MINIMUM	MAXIMUM	95 PCT CONF INT FOR MEAN
GRP1	19	1.7458 a	0.5277	0.1211	1.1100	2.8900	1.4914 TO 2.0001
GRP2	17	3.1653 a	1.8307	0.4440	1.4400	6.2700	2.2240 TO 4.1065
GRP3	18	2.2494 a	0.5617	0.1324	1.6000	3.5300	1.9701 TO 2.5288
GRP4	18	2.9628 a	1.2468	0.2939	1.5700	5.4800	2.3428 TO 3.5828
GRP5	18	2.3511 a	1.2660	0.2984	1.1700	5.3800	1.7216 TO 2.9807
GRP6	18	2.5461 a	1.4890	0.3510	1.5000	5.8800	1.8056 TO 3.2866
TOTAL	108	2.4903	1.2938	0.1245	1.1100	6.2700	2.2435 TO 2.7371
FIXED EFFECTS MODEL			1.2342	0.1188			2.2547 TO 2.7258
RANDOM EFFECTS MODEL			0.2098				1.9510 TO 3.0295

RANDOM EFFECTS MODEL - ESTIMATE OF BETWEEN COMPONENT VARIANCE 0.1792

TESTS FOR HOMOGENEITY OF VARIANCES

COCHRANS C = MAX. VARIANCE/SUM(VARIANCES) = 0.3596, P = 0.006 (APPROX.)
BARTLETT-BOX F = 7.278, P = 0.000
MAXIMUM VARIANCE / MINIMUM VARIANCE = 12.035

SECTION 5.3: SERIES I (BRETON)

VARIABLE VAR4 CBZPL
BY VARIABLE VAR3 TRT

ANALYSIS OF VARIANCE

SOURCE	D.F.	SUM OF SQUARES	MEAN SQUARES	F RATIO	F PROB.
BETWEEN GROUPS	11	7.9639	0.7240	3.908	0.0000
WITHIN GROUPS	165	30.5673	0.1853		
TOTAL	176	38.5312			

GROUP	COUNT	MEAN	STANDARD DEVIATION	STANDARD ERROR	MINIMUM	MAXIMUM	95 PCT CONF INT FOR MEAN
GRP1	14	1.8893 a	0.1633	0.0436	1.6200	2.1500	1.7950 TO 1.9836
GRP2	15	2.3627 b	0.6091	0.1573	1.5500	3.6300	2.0253 TO 2.7000
GRP3	15	2.8060 c	0.8769	0.2264	1.7200	3.9700	2.3204 TO 3.2916
GRP4	15	2.1093 a	0.4359	0.1125	1.5100	3.3800	1.8679 TO 2.3507
GRP5	15	2.2493 b	0.4192	0.1082	1.5300	3.0600	2.0172 TO 2.4815
GRP6	15	2.2333 a	0.2430	0.0627	1.9600	2.7600	2.0988 TO 2.3679
GRP7	14	2.3686 a	0.2360	0.0631	1.9400	2.6800	2.2323 TO 2.5048
GRP8	15	2.3733 b	0.2053	0.0530	2.0600	2.7100	2.2596 TO 2.4870
GRP9	15	2.4480 a	0.2865	0.0740	1.9400	3.1000	2.2893 TO 2.6067
GRP10	15	2.5167 b	0.5206	0.1344	1.9900	4.1600	2.2284 TO 2.8049
GRP11	14	2.2564 a	0.2798	0.0748	1.7200	2.6600	2.0949 TO 2.4180
GRP12	15	2.3520 a	0.2723	0.0703	1.7800	2.7200	2.2012 TO 2.5028
TOTAL	177	2.3331	0.4679	0.0352	1.5100	4.1600	2.2637 TO 2.4025
	FIXED EFFECTS MODEL		0.4304	0.0324			2.2692 TO 2.3970
	RANDOM EFFECTS MODEL			0.0640			2.1923 TO 2.4739

RANDOM EFFECTS MODEL - ESTIMATE OF BETWEEN COMPONENT VARIANCE 0.0365

TESTS FOR HOMOGENEITY OF VARIANCES

COCHRANS C = MAX. VARIANCE/SUM(VARIANCES) = 0.3504, P = 0.000 (APPROX.)
BARTLETT-BOX F = 6.967, P = 0.000
MAXIMUM VARIANCE / MINIMUM VARIANCE = 28.834

SECTION 5.3: SERIES II, SOIL MALMO